

**Gene therapy overexpressing Klotho for
Amyotrophic Lateral Sclerosis**

Sergi Verdés Franquesa

ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  <https://creativecommons.org/licenses/?lang=ca>

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  <https://creativecommons.org/licenses/?lang=es>

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  <https://creativecommons.org/licenses/?lang=en>



Gene therapy overexpressing Klotho for Amyotrophic Lateral Sclerosis

ACADEMIC DISSERTATION

To obtain the degree of PhD in Biochemistry, Molecular Biology, and
Biomedicine by the Universitat Autònoma de Barcelona (UAB), 2024

PhD Candidate

Sergi Verdés Franquesa

Thesis Supervisors

Dr. Assumpció Bosch Merino

Dr. Xavier Navarro Acebes

Group of Gene Therapy for
Neurometabolic Disorders

Group of Neuroplasticity and
Regeneration



This work was financially supported by:

- Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) affiliated with Secretaria d'Universitats i Recerca del Departament de Recerca i Universitats de la Generalitat de Catalunya, by means of a pre-doctoral FI grant (2019FI_B 00120) to Sergi Verdés Franquesa, co-funded by European Social Found.
- Project PID2020-116735RB-I00 (2021-2024) from Ministerio de Ciencia, Innovación y Universidades. "Gene therapy for amyotrophic lateral sclerosis directed to neuroprotection stimulation and modulation of neuroinflammation".
- Project Producte 2021 PROD 00030 (2023-2024) from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR). Generalitat de Catalunya. "Chronokine gene therapy, a new approach to tackle amyotrophic lateral sclerosis".
- Project 2021-SGR 00529 from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR). Generalitat de Catalunya. "Gene Therapy for diseases involving the Central Nervous System-TherCNS".

ACKNOWLEDGMENTS

Després de 7 anys al laboratori, 5 anys fent el doctorat, un canvi de projecte i uns mesos escrivint la tesi, voldria agrair a tots els que heu format part d'aquest camí. Sense vosaltres aquest doctorat i aquesta tesi no haguessin valgut la pena. Ha estat una etapa de creixement professional i personal, i el camí que hem construït junts ha marcat qui sóc a dia d'avui.

En primer lloc voldria agrair als meus directors de tesi, a l'Assumpció i el Xavi per tot el que heu fet per mi. Gràcies Assumpció per la confiança que des del principi has dipositat en mi, per la flexibilitat i la llibertat amb què m'has deixat treballar, i per totes les oportunitats que m'has brindat. Gràcies Xavi per tot el que he pogut aprendre de tu, tan científicament com personalment, pels teus savis consells, la teva sinceritat i també per la confiança. A la Mireia, per fer-ho tot més fàcil, el teu bon rotllo i simpatia, per la complicitat que tenim i per ser sempre lloablement fidel als teus principis. Al Miguel, pel teu bon humor, els teus consells i reflexions en els temes més abstractes, i pels granets de sorra que has aportat. Sou un veritable exemple de dedicació i vocació per la ciència.

En segon lloc m'agradaria agrair als companys de laboratori, que han compartit el dia a dia amb mi i hem viscut junts els millors però també els pitjors moments del doctorat. A tots els companys del CBATEG que m'han vist créixer des que vaig començar. A l'Ángela, amb qui vaig iniciar-me al laboratori i de qui he après tant, des de bon principi em vas transmetre la teva vocació per la nostra feina i això em va animar a seguir aquest camí. A la Sus, a qui mai podré estar prou agraït, ets tot un exemple per tots, per la teva amistat, companyonia, generositat i constància. Sempre disposada a ajudar i a anteposar el benestar dels teus companys.

A la Laia, la "floreta" del laboratori, que va transformar l'ambient des del primer dia que va arribar. Gràcies per irradiar sempre felicitat, transmetre bon rotllo i fer-ho tot més fàcil. Al Joan, sense qui aquest doctorat no hagués estat el mateix, per tota l'ajuda que m'has brindat, tots els moments viscuts, les nostres xerrades, per les teves bromes (que ja començo a pillar), per animar-nos i per treure'ns sempre un somriure. A la Laura, amb qui des que vam anar a Atenes hem forjat una gran amistat. Una de les persones amb qui comparteixo més complicitat i confiança, i a qui atorgo gran valor a la seva opinió. Gràcies sobretot per suportar-me aquests últims mesos d'escriptura i per tots els teus consells i la teva ajuda. Al Rubén, de bon grat puc afirmar que no només ha après ell de mi, sinó jo també molt d'ell. Per la teva bona energia i predisposició, per tot el que ens hem ajudat i hem viscut i el que ens queda per viure. A l'Alejandro pel seu esperit científic, la seva companyonia i les seves conyes. Tots vosaltres col·legues de feina que heu estat la meva família durant aquests anys i que us heu convertit en amics i persones essencials en la meva vida.

A les cares més noves, la Judith, la Maria, la Irina i el David, per la bona atmosfera que aporteu i perquè continueu propagant aquest bon ambient.

En especial agrair a la Judith, per tot el que m'has ajudat aquests últims mesos, per estar sempre predisposada a donar un cop de mà i per ser tan treballadora. Al Guillem, per implicar-se de valent en el projecte i aportar els seus coneixements, de ben segur t'espera una gran carrera. Als tècnics i companys de l'altre laboratori, al Javi, al Jorge, la Bea, i a la resta de companys de la UPV, per la seva ajuda i la facilitat de treballar amb ells. Particularment al Javi, per la seva valuosa contribució, per la implicació i preocupació que hi posa, i de qui també he tingut el plaer d'aprendre molts "truquillos".

Als que ja no estan al laboratori, la Txell, la Cris, el José, l'Ángel, la Belén, l'Andrea i el Marc. Gràcies Andrea per tots els moments de companys de poiata i d'escriptori, per les nostres converses i pels moments viscuts d'afterwork. A en Marc, com a model a seguir de perseverança, treball en equip i per la teva ajuda sempre desinteressada. Per totes les xerrades que teníem a últimes hores de la tarda, ja fossin científiques, personals o més abstractes, les quals alleujaven haver de treballar fins tard. Moments viscuts que es troben molt a faltar.

Per altra banda, també m'agradaria donar les gràcies a tots els companys del grup de Neuroplasticitat i Regeneració; Andrea, Bea, Bruno, Carla, Diego, Estef, Esther, Georgina, Jessica, Joan, Joana, Judith, Marc, Maria, Mónica, Néstor, Neus, Natàlia, Núria, Quino, Raquel, Rubèn, Sara, Sònia i Vera. Des del moment que vaig canviar de projecte em va fer sentir com un més d'una gran família, gràcies per ser tan bons companys, per tot el que m'heu ensenyat i m'heu ajudat.

Especialment a la Núria, per ser la primera en obrir-me les portes des de bon principi, per la complicitat que tenim i la facilitat amb què ens expliquem les coses, per la teva alegria i positivisme contagiosos i perquè una petita part d'aquesta tesi també et pertany. A la Joana, per ser tan amable i divertida, i per alegrar-nos a tots els dies amb els seus comentaris i accent morellà. A la Carla, per les hores compartides fent intratecals, tot i que finalment frustrades, per escoltar-nos i entendre'ns compartint les penes però també els bons moments. A la Maria, pel bon rotllo que desprends, pel teu optimisme i també per escoltar i entendre les meves frustracions. Espero que els cinc continuem celebrant la nostra amistat com hem fet fins ara.

També vull agrair a la Neus i a la Jessica la seva inestimable ajuda tècnica i per ser-hi sempre que he necessitat un cop de mà. Poder conèixer-vos i compartir aquesta etapa amb tots vosaltres ha fet que canviar de projecte a mitja tesi fos una de les decisions més encertades que he pres.

En tercer lloc vull agrair l'amistat que tenim amb la colla de Guissona, els "follets anaclets", a l'Arnau, l'Aida, l'Aina, l'Ares, el Dani, la Dània, el Jordi, la Júlia, la Rocío, el Roger i la Yasmin, que sempre m'han animat, s'han preocupat i interessat per mi durant aquesta etapa.

També a l'Ari, l'Aina F., la Marta, la Mar, la Clàudia i l'Ari C. Gràcies a tots pels nostres sopars, vermutos, calçotades, barbacoes, festes i viatges, i per ser el meu punt de suport i desconexió els caps de setmana al poble.

A la colla de la carrera, “els lliures d'esperit”. Fa deu anys que ens vam conèixer i heu esdevingut una segona família. Estic molt agraït per totes les trobades que hem compartit: les estades a cases rurals a l'estiu i al Nadal de cada any, els viatges inoblidables, els sopars tan animats i tot allò que hem fet per mantenir viva aquesta amistat tan especial que m'ha acompanyat al llarg dels meus anys de doctorat.

M'agradaria expressar un agraïment especial al Jordi, la Laura, la Marta, i la Cristina, els meus companys de pis durant aquesta etapa. Hem tingut la sort de viure junts molts anys i de compartir moments únics que recordarem sempre. Gràcies per tots els partits de pàdel, els sopars i beures, les caminades o sortides a córrer, les classes de bachata, les sessions de sèries i pel·lícules, les festes al pis, els petits moments quotidians i les anècdotes, que ara enyoro i aprecio encara més. Agraïxo també la vostra comprensió els dies que sortia més tard del laboratori o quan havia de treballar o acabar alguna cosa des de casa. Gràcies per adaptar-vos i tenir-me sempre en compte en aquests moments. La vostra amistat ha estat un suport essencial en aquesta etapa de la meua vida.

Finalment vull agrair a tota la meua família. Als meus tiets, a la Marta, al Jordi i a l'Alba, per ser-hi sempre i valorar el que tenim. A la meua germana Anna, la valenta de la família, que tot i estar a l'altra punta del món sempre està present. Gràcies per recordar-me, a la teua manera, l'orgullosa que estàs de mi. Continua seguint els teus somnis, fent-nos sentir orgullosos de com lluites per aconseguir tot allò que et proposes. Als meus pares, gràcies per estimar-me com ho feu, pel vostre suport incondicional, per ensenyar-me a valorar el que tinc i a donar-ho tot per aquells que m'estimen. Gràcies per ser el meu model a seguir, per ajudar-me a arribar fins aquí, per donar-me tot allò que vosaltres no vau poder tenir, per demostrar-me com ser perseverant, resistent i a esforçar-me pel que desitjo. Però sobretot per ensenyar-me a ser feliç i ser conscient de la sort que tinc de comptar amb tota la gent fantàstica que m'acompanya dia a dia i que ho ha fet durant aquesta etapa. Una etapa que acaba, però que en certa manera continua amb tots vosaltres, i que recordaré com una de les millors de la meua vida.

TABLE OF CONTENTS

ABSTRACT	1
ABBREVIATIONS.....	5
INTRODUCTION	13
1..Amyotrophic lateral sclerosis (ALS).....	15
1.1. Epidemiology and etiology of ALS	15
1.2. Phenotypic variation and clinical presentations	16
1.3. Pathophysiological mechanisms in ALS	17
1.3.1. Excitotoxicity	18
1.3.2. Impaired proteostasis, protein aggregation, and ER stress	19
1.3.3. Mitochondrial dysfunction	20
1.3.4. Oxidative stress	21
1.3.5. Cytoskeletal and axonal transport defects.....	22
1.3.6. Neuroinflammation and astrogliosis	22
1.3.7. Oligodendrocyte and Schwann cell dysfunction	24
1.3.8. Disturbances in RNA metabolism.....	25
1.3.9. Impaired DNA repair and nucleocytoplasmic transport.....	26
1.4. Determinants of motoneuron vulnerability	28
1.5. Skeletal muscle involvement in ALS	29
1.6. Forward or backward motoneuron degeneration	30
1.7. ALS experimental models	31
1.7.1. In vitro models.....	31
1.7.2. In vivo rodent models	32
1.8. Available therapeutic strategies for ALS.....	34
2..Gene therapy.....	35
2.1. Gene therapy overview	35
2.2. Viral vectors for gene therapy	37
2.3. Adeno-associated viral vectors (AAV)	38
2.3.1. AAV biology	38
2.3.2. Recombinant AAV viral vectors and expression cassettes	40
2.4. Gene therapy in ALS.....	43
2.4.1. Gene delivery.....	43
2.4.2. Gene silencing	45
SOD1 silencing.....	45
C9ORF72 silencing.....	46
FUS silencing	47
TDP-43 silencing	47
3..Klotho	48
3.1. Klotho gene and isoforms.....	49
3.2. Klotho mechanisms of action	51
3.1.1. Klotho in mineral metabolism	53

3.1.2. Inhibition of the insulin/IGF-1 pathway.....	53
3.1.3. Wnt/ β -Catenin signaling inhibition	54
3.1.4. Antioxidative effects of Klotho.....	54
3.1.5. Klotho's anti-inflammatory profile.....	55
3.1.6. Klotho in excitotoxicity.....	55
3.1.7. Klotho in ER stress and autophagy.....	56
3.1.8. The role of Klotho in myelination	56
3.1.9. Modulation of senescence.....	56
3.1.10. Klotho protection from cognitive decline	57
3.1.11. Muscle and myofiber regeneration depend on Klotho	58
3.2. Klotho and ALS: previous evidence	59
HYPOTHESIS AND OBJECTIVES	61
GRAPHICAL STUDY DESIGN.....	65
RESULTS.....	74
Chapter I: Validation of Klotho in models of motoneuron degeneration.	
Therapeutic overexpression of Klotho in the spinal cord of SOD1 ^{G93A} mice	76
1.1. Klotho decreases in models of MN degeneration and muscle atrophy	78
1.2. Klotho protects motoneurons in an in vitro model of motoneuron degeneration	79
1.3. Overexpression of Klotho in the spinal cord of SOD1 ^{G93A} mice	84
1.3.1. sKL partially preserves neuromuscular function in female SOD1 ^{G93A} mice.....	85
1.3.2. sKL preserves motor function in SOD1 ^{G93A} mice but fails to maintain body weight and muscle mass	86
Chapter II: Targeting skeletal muscle-specific overexpression of Klotho with systemic AAV8 vectors.....	
2.1. Overexpression of Klotho in muscles with AAV8 vectors	91
2.2. AAV8-Des-sKL preserves neuromuscular function in female SOD1 ^{G93A} mice.....	92
2.3. AAV8-Des-sKL boosts overall function in female SOD1 ^{G93A} mice.....	94
2.4. Muscular secretion of KL preserves MNs and NMJ, and decreases glial reactivity,.....	95
Chapter III: Enhanced Klotho delivery to muscles with the novel myotropic MyoAAV vector.....	
3.1. MyoAAV-Des-sKL therapeutic dose-finding study	100
3.2. MyoAAV-mediated overexpression of sKL before disease onset	102
3.2.1. MyoAAV-Des-sKL preserves neuromuscular function in SOD1 ^{G93A} mice.....	102
3.2.2. Muscle sKL overexpression induced by MyoAAV vectors improves motor performance, delays disease onset, and extends survival	106
3.2.3. Secreted KL overexpression protects spinal and cortical MNs, preserves NMJ, and reduces glial reactivity	109
3.3. MyoAAV-mediated muscle overexpression of Klotho at a symptomatic stage	112
3.3.1. Secreted KL overexpression slows down the decline in neuromuscular function of SOD1 ^{G93A} mice.....	113

3.3.2. Secreted KL muscle overexpression protects MNs and preserves NMJ while sustaining motor function in symptomatic SOD1 ^{G93A} mice	115
3.4. Widespread overexpression of sKL fails to preserve neuromuscular function in SOD1 ^{G93A} mice	116
Chapter IV: Transcriptomic and proteomic studies unravel Klotho mechanisms of protection in ALS	119
4.1. Overexpression of sKL in muscles alters gene expression profile in the spinal cord	121
4.1.1. Transcriptomic profiling of the spinal cord in sKL- and Null-treated mice	121
4.1.2. Protein expression patterns in the spinal cord of sKL and Null-treated mice	124
4.2. Overexpression of sKL in the muscles results in rectified gene expression patterns.....	128
4.2.1. Transcriptomic profile of the gastrocnemius muscle in sKL- and Null-treated mice	128
4.2.2. Proteomic analysis of the gastrocnemius muscle in sKL- and Null-treated mice	131
4.3. Integration of RNAseq and proteomics data in the spinal cord and gastrocnemius muscle ...	135
DISCUSSION	139
CONCLUSIONS	167
MATERIALS AND METHODS	172
1..DNA cloning techniques	174
1.1. Therapeutic construct and plasmid generation.....	174
1.2. Enzymatic restriction of plasmids or DNA products	176
1.3. Separation and purification of digested DNA fragments	176
1.4. DNA dephosphorylation and ligation	177
1.5. Competent <i>E.coli</i> TOP10 preparation.....	177
1.6. Bacterial transformation and plasmid amplification	178
2..Viral vectors	179
2.1. Generation of AAV vectors.....	180
2.1.1. Triple transfection and cell harvest.....	180
2.1.2. AAV purification	181
2.1.3. AAV quantification.....	182
2.1.4. Sterility and mycoplasma tests	183
3..In vitro cultures	183
3.1. Cell lines	183
3.1.1. Cell seeding.....	183
3.1.2. Cell passage and maintenance.....	184
3.1.3. Cell freezing.....	184
3.2. Spinal cord organotypic cultures (SCOCs).....	184
4..Animal experiments	185
4.1. Animal housing	185
4.2. hSOD1 ^{G93A} mice genotyping	186

4.2.1. Conventional PCR genotyping	186
4.2.2. Digital PCR for hSOD1 ^{G93A} copy number quantification	186
4.2.3. Real-time PCR for hSOD1 ^{G93A} copy number verification	187
4.3. Surgical procedures.....	188
4.3.1. Brachial plexus injury model	188
4.3.2. Viral vector intrathecal and intravenous administrations	188
4.4. Electrophysiological tests.....	189
4.4.1. Nerve conduction tests	189
4.4.2. Motor unit number estimation (MUNE).....	190
4.5. Behavioral tests	191
4.5.1. Rotarod test and clinical disease onset	191
4.5.2. Grip strength test	191
4.5.3. Treadmill test.....	191
4.5.4. Body weight and clasping monitoring.....	192
4.5.5. Survival analysis.....	192
4.6. Animal euthanasia, perfusion, and sample processing	192
5.. Histological analysis	193
5.1. Cresyl violet of spinal cord sections	193
5.2. Immunohistochemistry	194
5.2.1. SCOCs immunofluorescence staining and analysis.....	194
5.2.2. Immunohistochemistry of spinal cord slices	194
5.2.3. Immunohistochemistry of muscles and KL signal quantification	195
5.2.4. Immunohistochemistry and analysis of NMJ.....	196
5.2.5. Immunohistochemistry of brain sections and cortical neuron quantification	197
6.. ELISA	198
6.1. Protein extraction for ELISA	198
6.2. Protein quantification	198
6.3. Soluble Klotho ELISA	198
7.. RNA sequencing.....	199
7.1. RNA extraction	199
7.2. RNA library preparation, sequencing, mapping, and analysis	199
8.. Proteomic analysis	200
8.1. Protein extraction.....	200
8.2. Mass spectrometry/Proteomics and data analysis	201
9.. Statistical analysis	201
REFERENCES	204
APPENDIX.....	244
PATENT PUBLICATION	255

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a debilitating neuromuscular disease currently lacking effective treatments. The pathophysiological mechanisms involved in the degeneration of motoneurons (MNs) and muscle denervation are intricate and diverse. The key to enhancing functional outcomes in ALS lies in preventing axonal detachment from muscles, protecting MNs, and promoting reinnervation.

α -Klotho (KL) emerges as a pleiotropic chronokine with notable neuroprotective and myoregenerative properties, including anti-oxidative and anti-inflammatory effects, promoting myelination, protecting from excitotoxicity, and maintaining mitochondrial ultrastructure and function. In the present thesis, we have explored the therapeutic potential of KL overexpression in both the central nervous system and skeletal muscle in ALS. For this purpose, we first identified that the endogenous levels of secreted and transmembrane KL in these tissues were downregulated in two models of MN degeneration and muscle atrophy. Next, we investigated the protective effects of secreted and transmembrane KL against acute excitotoxicity using spinal cord organotypic cultures (SCOCs), revealing complete protection of spinal MNs from glutamate-induced excitotoxicity upon KL overexpression.

Capitalizing on the pleiotropic benefits of KL, we employed gene therapies based on adeno-associated viral vectors (AAV) to overexpress secreted KL in the spinal cord and skeletal muscles of the SOD1G93A mouse model of ALS. Our results indicate that CNS overexpression had a mild impact on neuromuscular preservation, only observed in females, but it improved motor function in both sexes. On the other hand, enhancing muscle secretion of KL protected MNs, mitigated astrocyte and microglial reactivity, and preserved neuromuscular junctions (NMJ). This intervention resulted in preserved muscle mass and neuromuscular function, improved locomotor function and strength, delayed disease onset, and prolonged survival. Importantly, even at a symptomatic stage, muscle overexpression of KL slowed down the progressive neuromuscular and motor decline characteristic of SOD1G93A mice.

Finally, we conducted comprehensive transcriptomics and proteomics in the spinal cord and gastrocnemius muscles, elucidating the primary mechanisms underlying the neuroprotective effects of KL in ALS. Overall, our findings offer compelling evidence that muscle secretion of KL can foster functional improvement in ALS, potentially paving the way for a novel avenue in the treatment of this devastating disorder.

ABBREVIATIONS

A

aa: Amino Acid
AAP: Adeno-Associated Virus-Associated Peptide
AAV: Adeno-Associated Virus
AAVS1: AAV Integration Site 1
ACTN3: α -actinin-3
Ad: Adenovirus
ADAR2: Adenosine Deaminase RNA Specific B2
AdV: Adenoviral Vector
AHC: Anterior Horn Cell
ALDH1L2: Aldehyde dehydrogenase 1 family member L2
ALS-FRS-R: ALS Functional Rating Scale-Revised
ALS-FTD: ALS with Frontotemporal Dementia
ALS: Amyotrophic Lateral Sclerosis
ALS2: Alsin
AMPA: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR: AMPA Receptor
AMPK: AMP-Activated Protein Kinase
ANKRD1: Ankyrin repeat domain-containing protein 1
AP2S1: AP-2 complex subunit sigma-1
APP: Amyloid Beta Precursor Protein
ASK1: Apoptosis Signal-Regulating Kinase 1
ASO: Antisense Oligonucleotide
ASS1: Argininosuccinate synthase 1
ATF4: Activating transcription factor 4
Atg5: Autophagy-related protein 5
ATG7: Autophagy-related 7 gene
ATP: Adenosine Triphosphate
ATPase: Adenosine 5'-TriPhosphatase
ATXN2: Ataxin-2
AXDND1: Axonemal dynein light chain domain-containing protein 1

B

BAC: Bacterial Artificial Chromosome
BBB score: Basso-Beattie-Bresnahan locomotor rating score

BBB: Blood-Brain Barrier
Bcl-2: B-cell lymphoma 2
BiP: Binding Immunoglobulin Protein

C

C9FTD/ALS: C9ORF72-linked Frontotemporal Dementia/ALS
C9ORF72: Chromosome 9 open reading frame 72
Ca²⁺: Calcium ion
CAG: Cytomegalovirus Immediate-early Enhancer/Chicken β -actin (promoter)
CAMK2A: Calcium/calmodulin-dependent protein kinase type II subunit alpha
Cb: combined
CB: cytomegalovirus enhancer/ β -actin promoter
CBA: chicken beta-actin
CBE: cytosine base editor
CDKN1A: Cyclin-dependent kinase inhibitor 1A (p21)
ChAT: Choline Acetyltransferase
CHL1: Cell Adhesion Molecule L1 Like
CHOP: C/EBP homologous protein
CMAP: Compound Muscle Action Potential
CMV: Cytomegalovirus (promoter)
CNS: Central Nervous System
COL19A1: Collagen type XIX α 1 chain
Cox: Cyclooxygenase
CpG: Cytosine-phosphate-Guanine
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CSF: Cerebrospinal Fluid
CT-1: cardiotrophin 1
CTNF: ciliary neurotrophic factor
CytC: Cytochrome C

D

DAO: D-Amino Acid Oxidase
DCN: deep cerebellar nuclei
DCTN: Dynactin

DDR: DNA Damage Response
DEG: Differentially Expressed Genes
Des: Desmin (promoter)
DIA: diaphragm
DKK1: Dickkopf WNT Signaling Pathway Inhibitor 1
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic Acid
DOK7: Downstream of Kinase 7
DPM3: Dolichol-phosphate mannosyltransferase subunit 3
DPR: Dipeptide Repeat
DYSF: Dysferlin

E

EAAT1-4: Excitatory Amino Acid Transporter 1-4
EAE: Experimental Autoimmune Encephalomyelitis
8OH2'dG: 8-Hydroxy-2'-deoxyguanosine
EMA: European Medicines Agency
ENA: 2'-O,4'-C-ethylene nucleic acids
EPHA4: Ephrin Type-A Receptor 4
ER: Endoplasmic Reticulum
ErbB2: Receptor Tyrosine-Protein Kinase erbB-2
ERK1: Extracellular Signal-Regulated Kinase 1
ESWR1: Ewing Sarcoma Breakpoint Region 1
ETC: Electron Transport Chain
EV: Extracellular Vesicle

F

fALS: Familial ALS
FCM: facial muscles
FC: Fold-change
FDA: Food and Drug Administration
FDR: False Discovery Rate
FEZ-1: Fasciculation and Elongation Protein Zeta-1
FGF: Fibroblast Growth Factor
FGF23: Fibroblast Growth Factor 23

FL: forelimb
FMN: facial motor nucleus
FOXOs: Forkhead Box O
FRET: Fluorescence Resonance Energy Transfer
FTD: Frontotemporal Dementia
FUS: Fused in Sarcoma

G

G-CSF: granulocyte-colony stimulating factor
G4C2: Guanine-4 Cytosine-2
GABA: Gamma-Aminobutyric Acid
GDH2: Glutamate Dehydrogenase 2
GDNF: Glial Cell-Derived Neurotrophic Factor
GDP: Guanosine Diphosphate
GDPH: Glyceraldehyde-3-phosphate dehydrogenase
GFAP: Glial Fibrillary Acidic Protein
GLT1: glial glutamate transporter 1
GLU: gluteus maximus
GluA2: subunit of the AMPA receptor
GluN2B: Glutamate Ionotropic Receptor NMDA Type Subunit 2B
GluR2: Glutamate Receptor 2
GM: Gastrocnemius muscle
GNK: Gankyrin
GO: Gene Ontology
GRIA1: Glutamate Ionotropic Receptor AMPA Type Subunit 1
GSEA: Gene Set Enrichment Analysis
GTP: Guanosine Triphosphate

H

H2-D1: Histocompatibility 2, D Region Locus 1
HAX-1: HCLS1-Associated Protein X-1
HBE: Heterochronic Blood Exchange
HEATR5B: HEAT repeat-containing protein 5B
HEK293: Human Embryonic Kidney 293 cells
HGF: hepatic growth factor

HIG1: Hypoxia-inducible gene 1 protein

His: Histidine

HL: hindlimb

hnRPN: Heterogeneous Nuclear
Ribonucleoprotein

HSPG: Heparan Sulfate Proteoglycan

HSV: Herpes Simplex Virus

I

i.c.: Intracerebral

i.c.m.: Intracisterna Magna

i.c.v.: Intracerebroventricular

i.l.: Intralingual

i.m.: Intramuscular

i.p.: Intrapleural

i.s.: Intraspinal

i.s.t.: Intrastratial

i.t.: Intrathecal

i.v.: Intravenous

Iba1: Ionized Calcium-Binding Adapter Molecule
1

ICap: internal capsule

IGF-1: Insulin-Like Growth Factor 1

IGF2: Insulin-Like Growth Factor 2

IL: Interleukin

InC: intercostal muscles

INF- α : Interferon- α

iPSCs: Induced Pluripotent Stem Cells

IR: Insulin Receptor

IRE1: Inositol-Requiring Enzyme 1

IRS: Insulin Receptor Substrate

ITR: Inverted Terminal Repeat

J

JAKMIP3: Janus kinase and microtubule-
interacting protein 3

jALS: Juvenile ALS

K

KAP3: Kinesin-Associated Protein 3

KASP3: Keratin-Associated Protein 3

Kb: Kilo Base

KCC2: Potassium-Chloride Cotransporter 2

KEEG: Kyoto Encyclopedia of Genes and
Genomes

KL: α -Klotho

L

LC-MS/MS: Liquid chromatography-mass
spectrometry/mass spectrometry

LIAS: Lipoic acid synthetase

LKB1: Liver Kinase B1

LMN: Lower Motoneuron

LPS: Lipopolysaccharide

LT: Longissimus thoracis

LV: Lentivirus

M

MAAP: Membrane-Associated Accessory
Protein

MAG: Myelin-Associated Glycoprotein

MAM: Mitochondria-associated membrane

MAP: Microtubule-Associated Protein

MAPK: Mitogen-Activated Protein Kinase

MBP: Myelin Basic Protein

MC: Motor cortex

MCP1: Monocyte Chemoattractant Protein 1

MCT1: Monocarboxylate Transporter 1

MCU: Mitochondrial calcium uniporter

MFAP4: Microfibril-associated glycoprotein 4

MICU: Mitochondrial calcium uptake protein

MIF-2: Macrophage migration inhibitory factor 2

MIF: Macrophage migration inhibitory factor

miRNA: MicroRNA

mKL: Membrane-bound α -Klotho

MMP-9: Matrix Metalloproteinase 9

MMP: Measurement of mitochondrial function
MN: Motorneuron
MND: Motor Neuron Disease
MnSOD: Manganese Superoxide Dismutase
MPC: Muscle Progenitor Cell
MRI: Magnetic Resonance Imaging
mRNA: Messenger RNA
MSC: Mesenchymal stem cells
mSOD1: Mutant Superoxide Dismutase 1
mtDNA: Mitochondrial DNA
MU: Motor Unit
MuSC: Muscle Stem Cell
MuSK: muscle-specific receptor tyrosine kinase
MYBPC2: Myosin-binding protein C, fast-type
MYH4: Myosin heavy chain 4
MYLK2: Myosin light chain kinase 2
MYOD1: Myogenic differentiation 1
MYOG: Myogenin
MYOT: Myotilin

N

NAb: neutralizing antibodies
NCAM1: Neural Cell Adhesion Molecule 1
NCOR1: Nuclear Receptor Corepressor 1
NCT: Nucleocytoplasmic Transport
NECTIN1: Nectin Cell Adhesion Molecule 1
NEK1: Never in Mitosis Gene A-Related Kinase 1
NES: Normalized Enrichment Score
NF-kB: Nuclear Factor Kappa-B
NFIX: Nuclear Factor I X
NFH: Neurofilament Heavy chain
NFL: Neurofilament Light chain
NFM: Neurofilament Medium chain
NFs: Neurofilaments
NG2: Neural Glial Antigen 2
NHP: Non-Human Primate

NLRP3: NOD-Like Receptor Family Pyrin Domain Containing 3
NLS: Nuclear Localization Signal
NMD: Nonsense-Mediated Decay
NMDA: N-methyl-D-aspartate
NMJ: Neuromuscular Junction
NNT: Nicotinamide Nucleotide Transhydrogenase
NO: Nitric Oxide
NOS-1: Nitric Oxide Synthase 1
NOS3: Nitric Oxide Synthase 3
NPC: neural progenitor cells
NQO1: NAD(P)H Quinone Dehydrogenase 1
NRF2: Nuclear Factor Erythroid 2-Related Factor 2
Nrg1-I: Neuregulin type I
Nrg1-III: Neuregulin 1 type III
Nrg1: Neuregulin 1
NS: neurological score
NSC-34: Motor Neuron-Like Cell Line
NT-3: Neurotrophin-3
NTFs: Neurotrophic Factors

O

O4: Oligodendrocyte Marker O4
Olig2: Oligodendrocyte Transcription Factor 2
OMN: Oculomotor Neuron
OXR1: Oxidation resistance 1

P

p-NF: phospho-neurofilament
p-TDP-43: Phosphorylated TDP-43
p21: Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A)
p53: Tumor Protein 53
p62: Sequestosome 1 (SQSTM)
PARK7: Parkinsonism Associated Deglycase
PARP: Poly(ADP-Ribose) Polymerase
PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PFN1: Profilin 1

PGC-1a: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha

PGK1: Phosphoglycerate Kinase 1 (promoter)

PHKG1: Phosphorylase kinase catalytic subunit gamma 1

PhMNs: phrenic motoneurons

Pi: Inorganic Phosphate

PI3: Phosphatidylinositol 3

pKL: Processed α -Klotho

PKLR: Pyruvate Kinase PKLR

PL: Plantar interossei muscles

PLCB4: Phospholipase C β 4

PLS: Primary Lateral Sclerosis

PMA: Progressive Muscular Atrophy

PMN: Progressive motor neuronopathy

PNS: Peripheral Nervous System

polyA: Polyadenylation signal

PPA: Primary Progressive Aphasia

Prp: Prion Protein (promoter)

Prx: Peroxiredoxin

PSM: Paraspinal muscles

PTH: Parathyroid Hormone

PTPRQ: Protein tyrosine phosphatase, receptor type Q

PU.1: PU-box binding protein 1

Q

QD: Quadriceps

R

rAAV: Recombinant Adeno-Associated Virus

RBP: RNA-Binding Protein

RET: Rearranged During Transfection (GDNF receptor)

RNA: Ribonucleic Acid

RNAi: RNA Interference

RNAseq: RNA Sequencing

ROAST: Rotation Gene Set Testing

ROS: Reactive Oxygen Species

RRAT: RNA regulator of axon terminal development homolog

rRNA: Ribosomal RNA

RSV LTR: Rous sarcoma virus long terminal repeat promoter

RV: Retrovirus

S

s.p.: Subpial

S100: S100 Calcium Binding Protein

SA β -Gal: Senescence-Associated β -Galactosidase

SaCas9: Streptococcus aureus Cas9

sALS: Sporadic ALS

SCOCs: Spinal Cord Organotypic Cultures

SD: Sprague Dawley rats

Sema: Semaphorin

SEPTIN7: Septin 7

SETX: Senataxin

SFP: spontaneous fibrillation potentials

SG: Stress Granule

shRNA: Short Hairpin RNA

siRNA: Small Interfering RNA

sKL: Secreted α -Klotho

Slc: Solute Carrier

SLN: Sarcolipin

SM: Skeletal Muscle

SMA: Spinal Muscular Atrophy

SNX10: Sorting nexin-10

SOD1: Superoxide Dismutase 1

SOD2: Superoxide Dismutase 2

ss: Single Strand

SUMO: Small ubiquitin-related modifier

SV40: Simian Virus 40

SYN: Synapsin I

SYT13: Synaptotagmin 13

T

3D: Three-Dimensional

t.p.: Transplant

TA: Tibialis Anterior muscle

TAF15: TATA-Binding Protein-Associated Factor 15

TANGO2: Transport and Golgi organization protein 2 homolog

TARDBP: Transactive response DNA-binding protein gene

TB: Triceps brachii muscles

TC: Thoracic muscles

TDP-43: TAR DNA-binding protein 43

TG: Tongue

Tg: Transgenic

TGF- β : Transforming Growth Factor- β

TGF β -R: Transforming Growth Factor- β Receptor

THA: DL-threo- β -hydroxyaspartic acid

TIAL1: TIA1 cytotoxic granule-associated RNA-binding protein-like 1

TIMM: Translocase of the Inner Membrane Mitochondrial

TMEM: Transmembrane Protein

Tmx2: Thioredoxin-related transmembrane protein 2

TNF α : Tumor Necrosis Factor Alpha

TNFAIP2: Tumor Necrosis Factor α -Induced Protein 2

TOMM: Translocase of the Outer Membrane Mitochondrial

TOP1: DNA topoisomerase 1

TOP3B: DNA topoisomerase 3- β

TPM1: Tropomyosin 1

TRIO: Triple functional domain protein

Trx: Thioredoxin

TS: Triceps surae muscle

TSC: Terminal Schwann Cells

TSC2: Tuberous Sclerosis Complex 2 (tuberin)

TSPAN8: Tetraspanin-8

TTC19: Tetratricopeptide repeat protein 19

TU: Transducing Units

TUBA4A: Tubulin Alpha 4a

TUBB3: β 3-tubulin

2D: Two-Dimensional

U

UCH-L1: Ubiquitin carboxyl-terminal hydrolase isozyme L1

UMN: Upper Motoneuron

Unc13: Unc-13 Homolog

UPR: Unfolded Protein Response

UTR: Untranslated Region

V

VACHT: Vesicular Acetylcholine Transporter

VAK1: Ventral Anterior Homeobox 1

VDAC1: Voltage-Dependent Anion Channel 1

VEGF: Vascular Endothelial Growth Factor

VP1,2 and 3: Viral Protein 1, 2 and 3

W

WPRES: Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

WT: Wild-Type

X

XBP-1S: X-box Binding Protein 1 Spliced

XPO5: Exportin-5

Y

YPEL2: Yippee-Like 2

Z

ZFP697: Zinc finger protein 697

INTRODUCTION

1. Amyotrophic lateral sclerosis (ALS)

1.1. Epidemiology and etiology of ALS

Amyotrophic lateral sclerosis (ALS), also known as Charcot disease or Lou Gehrig's disease, is a fatal neurodegenerative disorder of the motor system and the most frequent form of motoneuron diseases (MND), with an incidence of 3-5 per 100,000 persons-year, being also one of the most common rare diseases. The occurrence and prevalence of ALS rise with advancing age and varies with sex in sporadic cases, with an average age of diagnosis at 55 years old and an overall male-to-female ratio of 3:1. The cumulative lifetime risk in Europe and the United States is approximately 1 in 400 individuals (Kiernan et al., 2011; Taylor et al., 2016). The name refers both to the degeneration of motoneurons (MNs) of the primary motor cortex, brainstem, spinal cord, and descending corticospinal tracts (lateral sclerosis), and the secondary denervation and wasting of muscles (amyotrophy). Although ALS typically begins in the limbs, it has a highly heterogeneous clinical presentation depending on the populations of MNs involved and the skeletal muscles affected, causing different patterns of involvement. Symptoms include marked weakness and muscle atrophy, hyperreflexia and spasticity, cramps, and muscle twitches (fasciculations); in some cases, cognitive alterations and sensory dysfunctions may also appear (Fang et al., 2017; Rubio et al 2022). Patients usually die within 2-5 years from diagnosis, mostly because of respiratory failure (Wijesekera & Leigh, 2009).

Sporadic ALS (sALS) accounts for most of the cases, with a peak age of onset of around 60 years. About 5-10% of the cases are familial forms (fALS) caused by inherited mutations, most being autosomal dominant, and with an age of onset a decade earlier than for sporadic cases. Cases in patients younger than 25 years are designated as juvenile-onset ALS (jALS) and usually have an autosomal recessive inheritance. Mutations in more than 30 genes have been described as causative or associated with an elevated risk of developing the disease. The most prevalent fALS genetic causes, accounting for up to 70% of the familial cases, are mutations in the Cu/Zn superoxide dismutase 1 (*SOD1*), TAR-DNA binding protein (*TARDBP*), fused in sarcoma (*FUS*) and hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (*C9ORF72*) (Mead et al., 2023; Shaw, 2005).

Motoneurons can be categorized into two groups; upper motoneurons (UMNs) located in the cerebral cortex and lower motoneurons (LMNs) in the brain stem and spinal cord. The impact of both populations of MNs is a distinctive neuropathological hallmark of ALS, discriminatory from other MNDs. Neurodegeneration occurs in the corticospinal and corticobulbar tracts, with loss of large pyramidal neurons in the primary motor cortex, and in the anterior horns of the brainstem and spinal cord.

As spinal MNs begin to fail, they first display electrical irritability, resulting in fasciculations, weakness, and atrophy, with synaptic connections to muscles lost as they undergo degeneration. In contrast, the involvement of corticospinal motor neurons leads to muscle stiffness, spasticity, pathologic reflexes, and clonus (Fig. 1) (R. H. Brown & Al-Chalabi, 2017).

In the context of the initial onset of ALS, there are two prevailing hypotheses: one proposes that it originates as a distal axonopathy, while the other suggests that degeneration spreads in a corticofugal manner. In the first perspective, axonal retraction is thought to lead, in a “dying-back” pattern, to the death of MNs and subsequent muscle atrophy (Azzouz et al., 1997; Fischer et al., 2004). On the other hand, the second hypothesis debates a “dying-forward” process, suggesting that primary damage occurs in cortical MNs with hyperexcitability and spreads anterogradely along descending axonal projections (Braak et al., 2014). These differing viewpoints constitute the basis of the ongoing discourse surrounding the pathogenesis of ALS and may be relevant when addressing therapeutic strategies.

1.2. Phenotypic variation and clinical presentations

ALS manifests in a wide range of phenotypic variations, contingent on the specific groups of UMNs and/or LMNs first affected. Consequently, there is a gradual weakening of voluntary skeletal muscles involved in limb movement, speech (dysarthria), swallowing (dysphagia), and respiratory function. Oculomotor (OMNs) and Onuf's nucleus MNs are initially spared, preventing the deterioration of extraocular and sphincter muscles until the late stages of the disease. These varied clinical presentations are influenced by age, sex, and genetics and contribute to the complexity of the disease (Feldman et al., 2022). The most prevalent manifestation of ALS is the bulbar-onset, accounting for approximately one-third of the cases, closely followed by spinal-onset ALS. Also based on the regional distribution of involvement, patients less commonly present with primary lateral sclerosis (PLS), progressive muscular atrophy (PMA), respiratory onset, or hemiplegia (reviewed in Fig. 1). Most cases display a combination of UMN and LMN signs, which is known as classic ALS.

Progressive cognitive abnormalities characterized by behavioral changes are now identified to occur in 35-50% of patients with ALS, ultimately leading to dementia. At advanced stages of the disease, only a minor proportion of patients are free of neuropsychological deficits (Bersano et al., 2020; Crockford et al., 2018). Some patients present symptoms of frontotemporal dementia (FTD), aligned with postmortem findings of degeneration affecting the frontal and anterior temporal lobes of the brain (R. H. Brown & Al-Chalabi, 2017).

Approximately 10-15% of patients can be diagnosed with ALS-FTD when the criteria for behavioral variant FTD or primary progressive aphasia (PPA) are met. These cognitive and behavioral alterations bolster the idea that ALS is part of an extensive neurodegenerative disorder from FTD to pure ALS (Feldman et al., 2022; Masrori & Van Damme, 2020)

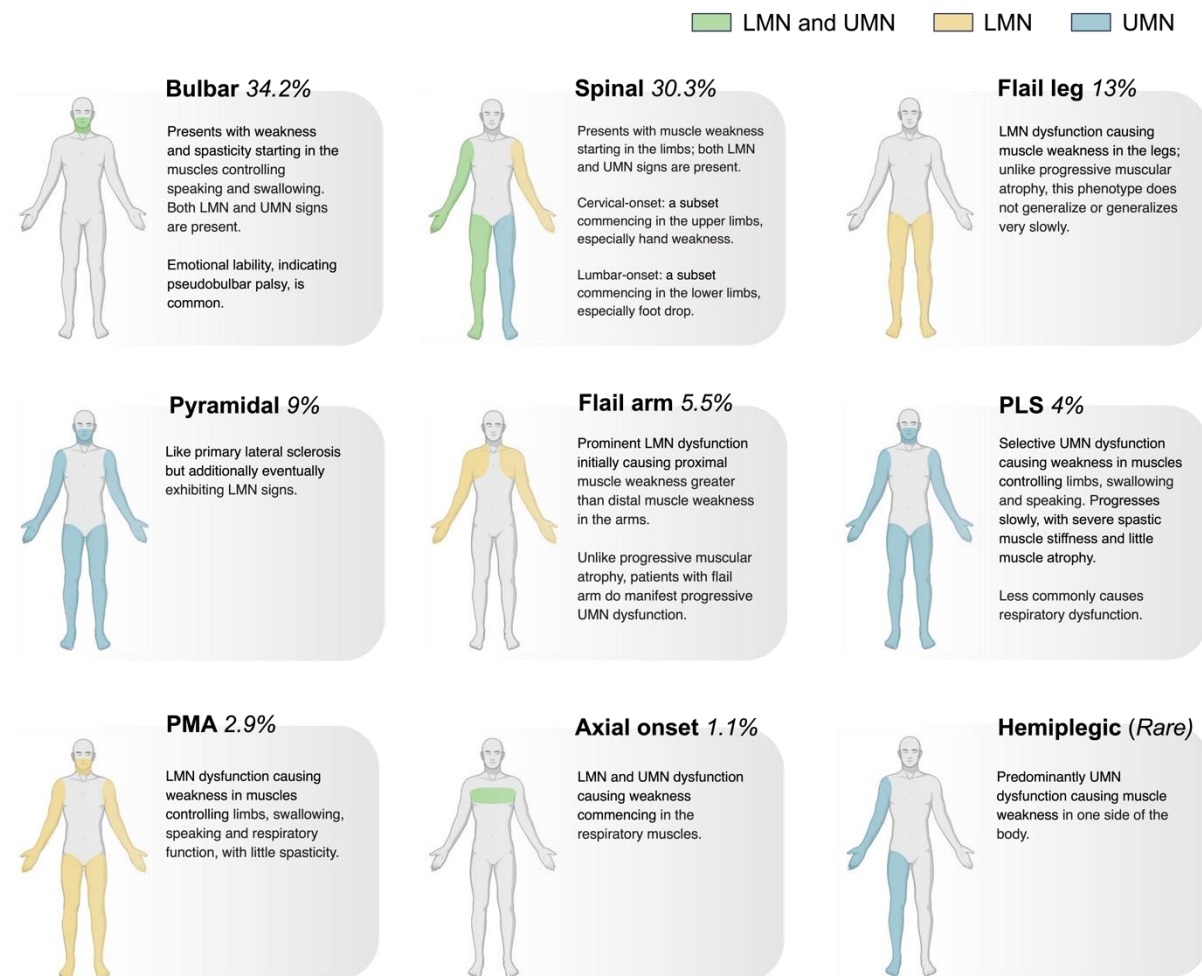


Figure 1. ALS clinical phenotypes. Individuals affected by ALS may exhibit indications of UMN dysfunction (depicted in blue), LMN dysfunction (yellow), or a combination of both UMN and LMN dysfunction (green). (Adapted from Feldman et al., 2022; Goutman et al., 2022).

1.3. Pathophysiological mechanisms in ALS

Several pathological mechanisms have been identified, mostly using ALS murine models, as underlying events of the disease process. These factors lead to selective MN death; however, it remains unknown whether there is an interplay between pathogenic mechanisms or if an earlier alteration triggers these downstream events that lead to neurodegeneration.

1.3.1. Excitotoxicity

As in other neurodegenerative disorders, excitotoxicity has been described as a major pathogenic mechanism in ALS, where increased glutamate levels have been found in patients' cerebrospinal fluid (CSF) (Perry, 1990; Spreux-Varoquaux et al., 2002). Interestingly, the first and one of the few drugs approved for ALS, Riluzole, acts by inhibiting glutamate release, a mechanism that seems to be shared in all forms of ALS.

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and acts through ionotropic and metabotropic receptors. Multiple studies point to the contribution of glutamate to MN degeneration, yet there is a lack of conclusive evidence to establish it as a primary driver of the disease. Excitatory mediators can cause neuronal damage when overactivating glutamate receptors, either by a deficient synaptic cleft clearance or because of hypersensitive receptors (Fig. 2G). Activation of glutamate receptors increases intracellular calcium (Ca^{2+}) and the activity of calcium-dependent nucleases, proteases, lipases, and reactive oxygen species (ROS)-generating enzymes that contribute to metabolic imbalances, mitochondrial abnormalities, and MNs injury (Arundine & Tymianski, 2003; Mancuso & Navarro, 2015).

The most susceptible MNs, damaged early in ALS, exhibit low calbindin and parvalbumin immunoreactivity and thus, show scarce calcium buffering capacity. MNs infrequently affected (oculomotor, trochlear, or Onuf's nucleus MNs) have higher expression of these proteins, highlighting the importance of excitotoxicity in the selective vulnerability of MNs in ALS (Alexianu et al., 1994).

Astrocytes are the primary controllers of extracellular glutamate by retrieving it from the synaptic cleft with the excitatory amino-acid transporter 2 (EAAT2). The expression and function of EAAT2 were found impaired in the CNS of MND patients (Bristol & Rothstein, 1996) and rodent models (Howland et al., 2002; Trotti et al., 1999).

Excessive stimulation of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors is considered a primary factor contributing to excitotoxicity (Van Den Bosch et al., 2006). Carriedo and colleagues (1996) demonstrated that cultured ALS MNs are particularly susceptible to excitotoxicity induced by AMPA receptors. That is because in ALS spinal MNs AMPA receptors have a lower proportion of the GluR2 subunit (Williams et al., 1997) and a defective post-transcriptional edition at the Gln/Arg⁵⁶⁸ site in the second transmembrane domain (Kawahara et al., 2004). The presence and modification of the GluR2 subunit renders the AMPA complex Ca^{2+} impermeable (Kwak et al., 2010; Williams et al., 1997). Thus, MNs in ALS are at a heightened risk of Ca^{2+} influx and are more exposed to excitotoxic

damage. This observation was additionally supported in mutant SOD1^{G93A} mice where the knock-in of GluR2 in cholinergic neurons promoted a substantial delay in the disease onset and mortality (Tateno et al., 2004).

The role of NMDA receptors has received considerably less focus despite its high expression in MNs. In cultured MNs (Urushitani et al., 2001) and in organotypic cultures of chick embryo spinal cords (Brunet et al., 2009), NMDA activation induces degeneration of MNs and the subsequent overload of mitochondrial Ca²⁺ and ROS production. Moreover, the application of sera from sALS patients to *in vitro* cultures expressing NMDA receptors induced their non-canonical activation. Lastly, mutated SOD1 protein aggregates and becomes integrated into membrane-forming pores, enabling the entry of calcium ions (Allen et al., 2012), potentially promoting excitotoxic harm to MNs.

1.3.2. Impaired proteostasis, protein aggregation, and ER stress

Another feature of ALS is abnormal protein deposits in spinal MNs. Aberrant or mutant proteins aggregate resulting in organelle dysfunction and neuronal damage (Fig. 2E). Ubiquitin-positive TDP-43 inclusions (Mackenzie et al., 2007; Neumann et al., 2006), Bunina-bodies (Okamoto et al., 2008), neurofilament-rich hyaline aggregates (Zhang et al., 1997), FUS aggregates (Hewitt et al., 2010) and SOD1 inclusions (Shibata et al., 1994) are found in different forms of ALS. Nevertheless, it remains unclear whether these inclusions have protective or detrimental roles in ALS (Mancuso & Navarro, 2015).

The accumulation of these misfolded proteins activates the endoplasmic reticulum (ER) stress response, which triggers signaling pathways collectively termed Unfolded-Protein Response (UPR). UPR is activated to cope with ER stress, provide adaptative responses for survival, and return to homeostasis (Hetz et al., 2012). However, if the protein-folding defect is not corrected, excessive ER stress and prolonged UPR lead to C/EBP homologous protein (CHOP) mediated apoptotic cell death of MNs and glial cells (Ito et al., 2009). UPR activation markers are present in the CSF and spinal cord of ALS patients (Atkin et al., 2008). Pharmacological alleviation of ER stress slows disease progression and attenuates manifestations in ALS animal models, whereas enhancing ER stress promotes disease manifestations (Saxena et al., 2009). ALS and FTD patients present neuronal and glial intracellular cytoplasmatic inclusions of TDP-43 (Neumann et al., 2006; Zhang et al., 2008). This phenomenon has not only been described in patients with TARDBP mutations but also in sALS and fALS patients (Sreedharan et al., 2008), except for those with SOD1-related ALS (Mackenzie et al., 2007). As a nuclear protein, TDP-43 is usually located within the nucleus, however nuclear loss and redistribution to the cytoplasm happen early in ALS.

Similarly, cytoplasmic inclusions containing mutated FUS protein are present in FUS-related familial ALS (Hewitt et al., 2010). Both TDP-43 and FUS are RNA-binding proteins, suggesting that defects in these proteins can lead to binding propensity and the accumulation of protein aggregates with the aberrant formation of stress granules (SG) in the cytoplasm. Persistent assembly of SG is cytotoxic and has been implicated as a driver of numerous neurodegenerative diseases (Lambert-Smith et al., 2022). Moreover, TDP-43 and FUS pathological aggregates can spread like a prion and propagate cell-to-cell, in a seed-dependent and self-templating manner (Chen et al., 2019; Jo et al., 2020).

Hexanucleotide GGGGCC (G_4C_2) repeat expansions in the *C9ORF72* locus are the most prevalent cause of fALS. G_4C_2 translate into five different dipeptide repeats (DPRs) (Ash et al., 2013; Mori et al., 2013). DPR proteins aggregate and interact with RNA processing factors and become toxic (Haeusler et al., 2014). For instance, DPR accumulation impairs the structural integrity of the nucleus and disrupts the nuclear membrane, causing the mislocalization of nuclear-cytoplasmic transport proteins (Ryan et al., 2022). The precise function of the *C9ORF72* protein remains unknown; however, it is likely to regulate membrane trafficking due to its structural resemblance to endosomal trafficking proteins that bind GDP-GTP exchange factors for RAB GTPases (Levine et al., 2013). Additionally, *C9ORF72* contributes to regulating the initiation of autophagy, and disruption of this role could potentially be associated with the appearance of ubiquitin⁺, p62⁺, and TDP43⁻ in the cerebellum and hippocampus, which distinguish *C9ORF72* cases from other ALS/FTD forms (Webster et al., 2016).

In short, compromised proteostasis within cells, triggered by the accumulation of toxic inclusions and aggregates and defective proteasomal and autophagic degradation systems, is closely linked to other ALS pathological mechanisms and plays an important role in MN degeneration.

1.3.3. Mitochondrial dysfunction

Mitochondrial impairment is one of the earliest events in ALS, even before disease onset, and has been extensively described in patients. Reduced activity of the electron transport chain (ETC) complexes, decreased ATP production, reduced and higher frequently mutated mitochondrial DNA (mtDNA) have been reported in ALS patients (Fig. 2H) (Borthwick et al., 1999; Hirano et al., 1984; Vielhaber, et al., 2000; Wiedemann et al., 1988, 2002). The exact mechanisms that disrupt mitochondrial function in ALS are still unclear. Several studies in the SOD1^{G93A} mouse model suggest that mSOD1 aggregates interfere with mitochondrial protein translocation (Parone et al., 2013).

Calcium influx into the mitochondria is impaired long before symptoms' onset for spinal and cortical MNs in SOD1^{G93A} and SOD1^{G85R} mice (Damiano et al., 2006; Fuchs et al., 2013). This reduction in mitochondrial Ca²⁺ loading capacity might be attributed to the downregulation and decreased efficacy of mitochondrial Ca²⁺ uptake proteins. Mitochondria also play a critical role in regulating apoptosis, as the opening of the permeability transition pore and the subsequent release of cytochrome C (CytC) are essential steps for initiating the activation of the caspase cascade. The B-cell lymphoma 2 (Bcl-2) anti-apoptotic protein avoids CytC release by stabilizing the mitochondrial membrane. Wild-type SOD1 can protect cells against apoptosis by interacting with Bcl-2, however, large mSOD1 aggregates in the ALS spinal cord entrap Bcl-2 and deplete MNs of this anti-apoptotic protein (Pasinelli et al., 2004).

Besides mSOD1, other ALS-causing genes have been reported to interfere with mitochondrial function. TDP-43 accumulates in the mitochondria of ALS patients, binding to mRNA encoding the respiratory chain complex subunits and promoting its disassembly (Wang et al., 2016). DPRs in C9ORF72-ALS models have been shown to adhere to mitochondrial ribosomal proteins and alter mitochondrial function, compromising oxidative stress and DNA damage (Lopez-Gonzalez et al., 2016). Overall, MNs are large cells with high energetic demand and are especially affected by mitochondrial dysfunction.

1.3.4. Oxidative stress

Oxidative stress goes hand in hand with neurodegeneration and is a key hallmark of MND. It occurs when there is an imbalance between the production and removal of reactive oxygen (ROS) and nitrogen (RNS) species (Fig. 2H). SOD1-depleted mice neither develop overt motor deficits nor show pathological signs, suggesting that degeneration might occur because of mSOD1 deposits rather than because of a loss of function. Nonetheless, after neuronal injury, SOD1 is required for MN survival and to overcome the accumulation of reactive oxygen species (ROS) (Reaume et al., 1996). Patients have increased ROS- and RNS-damaged proteins, lipids, DNA, and RNA in the CSF and spinal cord (Aoyama, 2000; Beal, 1997; Tohgi, 1999). Oxidation of mRNA species greatly decreases translation of proteins and mainly occur in MNs and oligodendrocytes in the spinal cord at an early stage of the disease (Chang et al., 2008).

As stated earlier, SOD1, TDP-43, and C9ORF72 DPRs can penetrate the mitochondria and interfere with the normal functioning of the respiratory chain, increasing the production of ROS/RNS (Liu et al., 2004; Lopez-Gonzalez et al., 2016; Wang et al., 2016). This establishes a connection between oxidative stress, mitochondrial dysfunction, and calcium homeostasis.

1.3.5. Cytoskeletal and axonal transport defects

MNs feature remarkably long axons, which can reach up to one meter within the human body to connect the neuronal cell body and distal synaptic sites. Ample evidence implicates defects in axonal transport in ALS disease mechanisms (Fig. 2I). Postmortem histological analysis in fALS patients revealed accumulations of neurofilaments (NFs) and cytoskeletal proteins, organelles, and mitochondria in proximal axons and perikarya of MNs (Hirano et al., 1984; M. L. Schmidt et al., 1987). The most prevalent fALS have been linked to alterations in intracellular trafficking (Burk & Pasterkamp, 2019) and ALS-causative mutations have been described in cytoskeletal and endosome trafficking genes such as profilin 1 (*PFN1*), dynactin (*DCTN*), tubulin α -4A (*TUBA4A*) or alsin (*ALS2*).

NF-overexpressing mice develop MND resembling ALS (Collard et al., 1995) and SOD1^{G93A} mice show impaired slow and fast axonal trafficking and accumulation of NFs (Tu et al., 1996; Zhang et al., 1997) as well as a notable decrease in dynein-mediated retrograde axonal transport. Anterograde axonal transport is also impaired in ALS as mSOD1 associate and sequester kinesin-associated protein 3 (KAP3) within the spinal cord ventral horn. This sequestration leads to a reduction in choline acetyltransferase (ChAT) within terminal axons, possibly explaining synaptic deficits (Tateno et al., 2009). Furthermore, several misfolded forms of SOD1 interact with tubulin and the ability to impact microtubule stability, worsened by increased Ca²⁺ that induce microtubule depolymerization (Kabuta et al., 2009; Furukawa & Mattson, 1995).

Mitochondria are unevenly distributed or forming clusters throughout axons in ALS MNs (De Vos et al., 2007) and in several mSOD1 (Bilsland et al., 2010; Magrané et al., 2012) and TDP43 murine models (Magrané et al., 2014). Glutamate receptors are also accumulated in post-synaptic densities of human induced MNs (Shi et al., 2018). These findings further support the cooperative role between pathological mechanisms.

Despite these indications, it is still unclear if impaired axonal transport is sufficient to instigate MN degeneration, as other studies propose that axons can persist despite notable dysfunction in axonal transport (Fischer et al., 2004; Marinković et al., 2012).

1.3.6. Neuroinflammation and astrogliosis

Even though ALS primarily impacts MNs, numerous studies have highlighted the involvement of neuroglia in the disease (Fig. 2F). In pathological conditions, reactive microglia and astroglia and the infiltration of monocytes and lymphocytes contribute to the worsening of neurodegeneration (Zhao et al., 2017). Transgenic rodent models have proved that overexpression of mSOD1 leads

to immune dysregulation and that glial cells become increasingly activated as the disease progresses (Henkel et al., 2009; Hensley et al., 2006). More recently, several studies have revealed the mechanistic processes underlying microglial activation in mouse models associated with TDP-43 (Xie et al., 2022) and C9ORF72 (O'Rourke et al., 2016), as well as in human C9ORF72 induced pluripotent stem cells (iPSC)-derived microglial cells (Banerjee et al., 2023; Vahsen et al., 2023).

High concentrations of protein aggregates activate microglia and astrocytes, prompting the secretion of proinflammatory signals (Kuhle et al., 2009; Zhao et al., 2015, 2017). As the disease progresses, the continuous accumulation of aberrant mutated proteins switches microglia to a neurotoxic state enhancing the secretion of Tumor Necrosis Factor α (TNF α), interleukin-6 (IL-6), superoxide, nitric oxide (NO), and other harmful cytokines and factors, inducing further MN injury (Beers & Appel, 2019).

MN degeneration in ALS has been demonstrated to be non-cell-autonomous with toxicity deriving from the combination of damaged neighboring cells, as restricting mSOD1 expression to neurons failed to induce MN cell death or motor impairment (Lino et al., 2002; Pramatarova et al., 2001). On the other hand, specific deletion of mSOD1 in MNs did not prevent MN degeneration (Boillée et al., 2006; Miller et al., 2005; Ralph et al., 2005). These methods prolonged survival by delaying the onset of the disease but did not decelerate the progression of the disease once started. These experiments established that expression of mSOD1 within individual MNs is not enough to trigger cell-autonomous degeneration.

In contrast, microglia seem to be determinant players of rapid disease progression. Removing mSOD1 specifically from microglia and macrophages had barely any effect on disease onset but slowed down disease progression and extended survival (Boillée et al., 2006; Ralph et al., 2005). Similarly, in a back-to-back study, birth transplantation of non-mutated myeloid cells to SOD1^{G93A}/PU.1^{-/-} mice (unable to produce their myeloid lineage) showed that replacement of microglia had also no effect on disease initiation but slowed the progression. Importantly, the introduction of myeloid cells expressing mSOD1 into control PU.1^{-/-} animals did not result in the development of MND. This underscores that mutant SOD1-expressing macrophages/microglial cells cannot singly trigger MN death (Beers et al., 2006).

Astrocytes are essential partners of MNs, as they play a vital role in providing nutrients, maintaining ion balance, and recycling the neurotransmitter glutamate. Again, limited expression of mSOD1 in this cell type does not result in MN degeneration (Gong et al., 2000). Nonetheless, selective reduction or absence of mSOD1 in astrocytes delays disease onset and prolongs the early phase of disease progression (Lepore et al., 2008; Wang et al., 2011; Yamanaka et al., 2008). This is associated with a later activation of microglia, indicating functional communication between

astrocytes and microglia. Furthermore, co-culturing astrocytes from both familial and sporadic ALS patients exhibit toxicity toward MNs, accompanied by an increase in the expression of inflammatory genes (Haidet-Phillips et al., 2011). Interestingly, ALS astrocytes exhibit selective toxicity towards MNs, as they do not trigger neurodegeneration in GABAergic (Gamma-aminobutyric acid producer) or dorsal root ganglion neurons (Di Giorgio et al., 2008; Madill et al., 2017; Nagai et al., 2007).

1.3.7. Oligodendrocyte and Schwann cell dysfunction

Oligodendrocytes and Schwann cells provide neurons with metabolic support and protection from environmental insults (Valori et al., 2014). Abnormalities related to oligodendrocytes, oligodendrocyte precursors, and myelin are common features of ALS (Kang et al., 2013; Kim et al., 2019; Zhou et al., 2017).

Degeneration of gray matter oligodendrocytes can be detected in SOD1^{G93A} mice well before the symptoms' onset. This degeneration also involves newly formed oligodendrocytes failing to reach maturity, which consequently leads to a gradual process of demyelination (Fig. 2B). The targeted removal of mSOD1 from NG2⁺ oligodendroglia significantly delayed disease onset and prolonged survival of the SOD1^{G93A} mice (Kang et al., 2013).

Moreover, oligodendrocytes offer direct energetic metabolic support to MNs' axons by supplying lactate through the monocarboxylate transporter 1 (MCT1). MCT1 is downregulated in ALS and contributes to axonal damage (Y. Lee et al., 2012). Schwann cells are responsible for myelinating individual axons in the peripheral nervous system (PNS). They are essential for proper synapse formation, impulse propagation, and the maintenance and repair of axons.

Schwann cells are reported to influence MN regeneration accuracy, adapting to the continuous denervation-reinnervation and guiding motor axons (Gorlewicz, 2009; Madison et al., 2011). Specific expression of mutant SOD1^{G93A} in Schwann cells did not produce differences in locomotion or neuronal degeneration and were resistant to mSOD1 aggregation (Turner et al., 2010). On the contrary, reducing the production of mSOD1 within Schwann cells notably hastens the progression of the disease. This suggests that an oxidative cascade is triggered within ensheathed axons during disease progression, and this process can be ameliorated through increased dismutase activity (Lobsiger et al., 2009).

Perisynaptic or terminal Schwann cells (TSC) are specialized glial cells found at the neuromuscular junction (NMJ). They have a specific role in the development, functioning, maintenance, and repair of synapses (Fig. 2A). TSCs play a prominent role in initiating and guiding collateral sprouting, a

process in which surviving MNs send axon terminals to denervated endplates attempting to reinnervate the muscle (Son & Thompson, 1995). Collateral sprouting is impaired in ALS (Martineau et al., 2020), and cannot compensate for functional deficits (Frey et al., 2000; Mancuso et al., 2011). Previous results from our groups show the importance of promoting collateral sprouting to improve functional recovery in SOD1^{G93A} mice (Mancuso et al., 2016).

1.3.8. Disturbances in RNA metabolism

The discovery of disease-associated mutations in *TARDBP* (Kwiatkowski et al., 2009; Vance et al., 2009) and *FUS* (Kwiatkowski et al., 2009; Vance et al., 2009) marked the initiation of a paradigm shift, emphasizing RNA metabolism abnormalities as a core pathological pathway in ALS and FTD (Fig. 2D). Additional ALS-linked mutations in RNA-binding proteins (RBPs) as ataxin-2 (Elden et al., 2010), hnRNPs (Beijer et al., 2021), angiogenin (Greenway et al., 2006), *TAF15* (Couthouis et al., 2011), senataxin (Chen et al., 2004) or *ESWR1* (Couthouis et al., 2012) further suggest a disruption in RNA homeostasis in ALS.

TDP-43 and FUS can interact with DNA, as well as RNA, and are certainly involved in a diverse array of cellular processes that are dependent on RNA metabolism. Typically, RNA-binding proteins are predominantly found in the nucleus. However, in ALS, they undergo mislocalization with an accumulation in the cytosol and a depletion in the nucleus (Mackenzie et al., 2007). This leads to the disruption of transcription or splicing events of their target RNAs, many of them related to neuronal integrity (Amlie-Wolf et al., 2015; Lagier-Tourenne et al., 2012; Zhou et al., 2014). The hyperassembly of TDP-43, FUS and hnRNPs (heterogeneous nuclear ribonucleoproteins) generates cytoplasmatic SG or non-dynamic RNA granules that fail to disassemble (Fig. 2E), generating fibrils that disrupt nuclear-cytoplasmic transport of mRNA and alter the transcriptional process of genes crucial for MN homeostasis (Kim et al., 2013; Kwiatkowski et al., 2009; Neumann et al., 2006c; Vance et al., 2009). The absence of nuclear TDP-43 also promotes the use of cryptic splice sites, which can lead to a decrease in the abundance of properly spliced mRNAs encoding proteins (Ling et al., 2015). The depletion of FUS or hnRNP from the nucleus results in similar abnormalities as those associated with TDP-43 loss, albeit each of these RNA-binding proteins is linked to the depletion of distinct sets of mRNAs.

In addition, when TDP-43 or FUS are lost, there is a decrease in miRNA expression (Dobrowolny et al., 2021; Emde et al., 2015; Zhang et al., 2013). Several miRNAs are involved in the maintenance of NMJs, suggesting that MNs could be particularly vulnerable to disruptions in miRNA biogenesis (Valdez et al., 2014; Williams et al., 2009). Concerning the expansion of hexanucleotide repeats in the *C9ORF72* gene, these sequences trigger the creation of stable

parallel monomeric and multimeric G-quadruplex structures. These structures rapidly interact with RNA processing factors (Haeusler et al., 2014; Reddy et al., 2013), resulting in the sequestration of RNA-binding proteins and forming nuclear RNA foci, potentially inducing direct RNA neurotoxicity (Cooper-Knock et al., 2014; Lee et al., 2013).

1.3.9. Impaired DNA repair and nucleocytoplasmic transport

Non-dividing cells like MNs are particularly prone to DNA damage, which can lead to cell death if unrepaired. Free oxygen radicals, increased Ca^{2+} concentrations, protein aggregates, and other insults are well-known inducers of DNA damage (Fig. 2C).

Evidence of DNA damage has been observed in pathological studies, including elevated levels of 8-hydroxy-2'-deoxyguanosine (8OH2'dG) in the CNS, plasma, urine, and CSF from ALS patients (Ferrante et al., 2002; Kok et al., 2021). Other findings have also shown elevated apurinic/apyrimidinic DNA sites, which indicate the loss of purine/pyrimidine bases from the DNA strand, as well as the activation of the DNA damage response (DDR). Similarly, several ALS-linked genes, including *TARDBP*, *FUS*, *NEK1*, or *SETX*, are known to regulate DNA repair and DDR (Bogdanov et al., 2000).

The G4C2 repeat expansion and TDP-43 can lead to the formation of R-loops, which are DNA-RNA hybrid structures. These structures bind to the ribonuclear proteins, causing nucleolar stress and enhancing the susceptibility to DNA damage and genome instability (Giannini et al., 2020; Haeusler et al., 2014). Moreover, DPR proteins are rich in positively charged amino acids (aa) that bind to DNA/RNA and displace DNA-binding proteins, profoundly affecting gene expression (Lafarga et al., 2021; Zhang et al., 2019).

In line with these findings, structural irregularities were observed in the nuclear envelope architecture in C9ORF72 ALS (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015). A developing hypothesis suggests that the abnormal attraction of nuclear-cytoplasmic transport (NCT) factors to SGs disrupts NCT, ultimately contributing to neurodegeneration (Zhang et al., 2018). The emerging picture is that DNA damage, impaired repair, and nuclear instability are common pathological features across ALS subtypes, however, a causal link to MN loss is not yet evident.

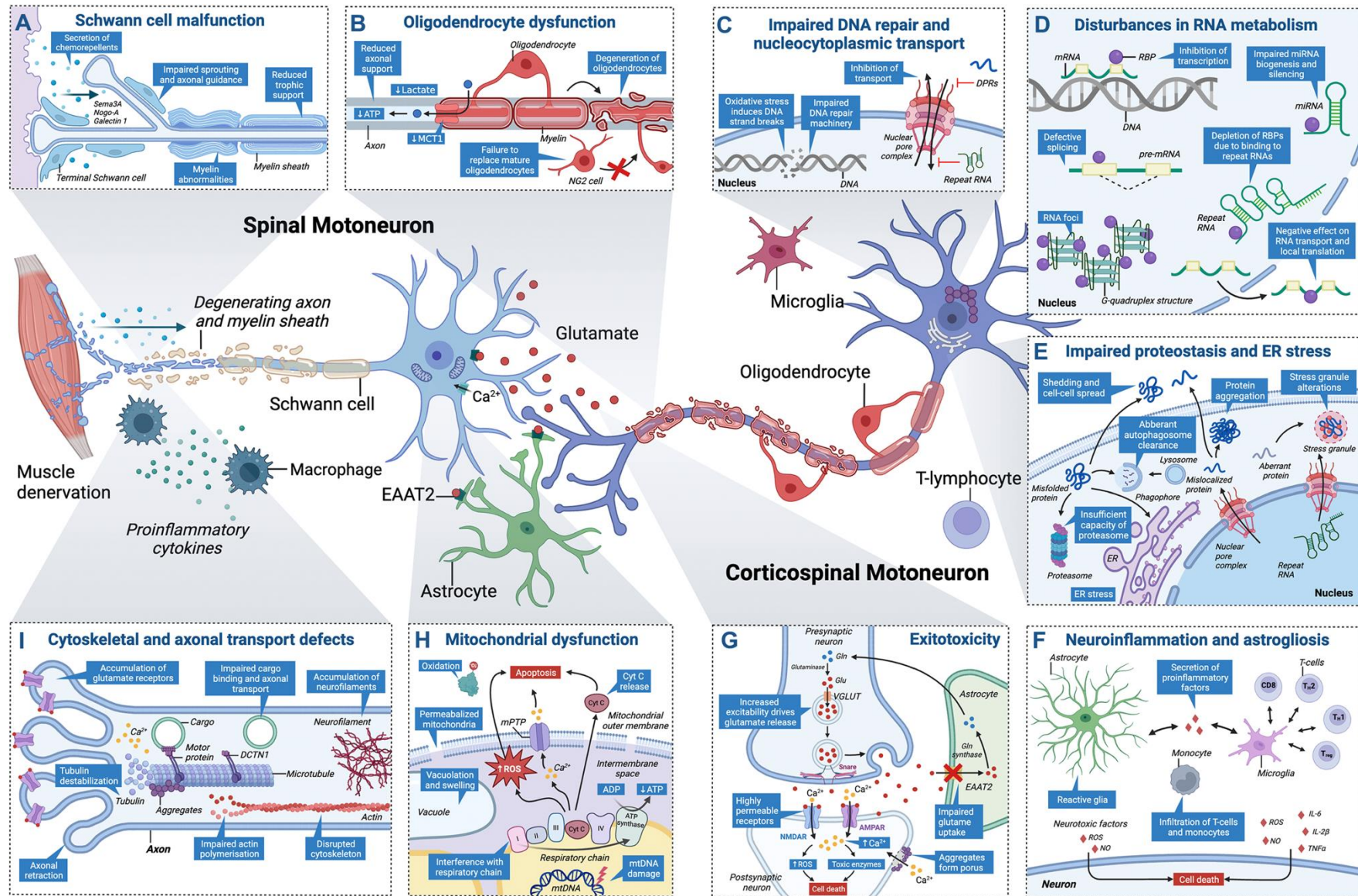


Figure 2. Molecular pathophysiological mechanisms of Amyotrophic lateral sclerosis. (Designed with Biorender. Adapted from Van Damme, et al. 2016)

1.4. Determinants of motoneuron vulnerability

ALS pathophysiological mechanisms converge in MN degeneration regardless of the origin of the disease. Several particularities make MNs more susceptible to degeneration than other types of neurons. First, MNs stand out from other types of neurons due to their unique morphological characteristics. They are notably large, with a diameter of approximately 50-60 μm , and possess exceptionally long axons. To maintain their functions and transport cargo along their axons they require a significant metabolic supply and are more prone to oxidative stress.

Second, large fast fatigable motor units (MUs) are more vulnerable than highly excitable small MU to degeneration. Fast-firing MNs in the SOD1^{G85R} mouse model were no longer detectable by the time mice exhibited motor impairment (Hadzipasic et al., 2014). Fast-twitching muscles innervated by fast fatigable MNs, such as the tibialis anterior (TA), have prompt denervation, whereas muscles composed mainly of slow MU are not affected until the late stages. OMNs that are initially spared in ALS are smaller, innervating only around 5 muscle fibers, in contrast to spinal MNs that can innervate up to 300 fibers, proving that the size of MNs and MU are determinants of ALS vulnerability.

Third, the global transcriptional profile of OMNs differs from spinal MNs and reveals preferentially enriched transcripts for each population. GABA receptor subunits, insulin-like growth factor 2 (*IGF-2*), parvalbumin, calbindin, and osteopontin are persistently expressed in OMNs, whereas spinal vulnerable MNs are enriched in metalloproteinase-9 (*MMP-9*) and *EPHA4* (Comley et al., 2015; Hedlund et al., 2010). The higher levels of the calcium buffering proteins could render this subtype of MNs more impervious to Ca^{2+} (Alexianu et al., 1994b). On the other hand, inhibiting *MMP-9* function in mSOD1 mice led to a notable delay in muscle denervation. Expression of *EPHA4*, a receptor in the ephrin axonal repellent system, showed an inverse correlation with age of disease onset and survival. Suppression of *EPHA4* signaling enhances survival and mitigates axonopathy (Van Hoecke et al., 2012).

Fourth, the intrinsic low Ca^{2+} buffering capacity of MNs added to their elevated Ca^{2+} permeability provides clues to why excitotoxicity and ER stress can have devastating effects on these cells. (Kwak et al., 2010; T. L. Williams et al., 1997). Fifth, several studies have indicated an imbalance between excitatory and inhibitory inputs affecting the synaptic connections with MNs (Sunico et al., 2011). The loss of inhibitory interneurons in the motor cortex of ALS patients first reinforced the evidence of cortical hyperexcitability (Nihei et al., 1993). Slow-resistant MNs with high excitability experience the fastest depolarization but have a slower firing rate. OMNs also exhibit a higher excitability compared to spinal MNs. Indeed, elevating the excitability of fast-fatiguing MNs before symptoms reversed the accumulation of mSOD1. Conversely, partially antagonizing MN

excitability promoted the aggregation of mSOD1, although decreased Ca^{2+} levels reduced ER stress (Saxena et al., 2013). Another dysregulated protein contributing to the disturbance between excitatory and inhibitory functions is the potassium-chloride co-transporter 2 (KCC2), responsible for keeping intracellular chloride levels low (Fuchs et al., 2010; Mòdol et al., 2014). Overall, a consensus view regarding the exact function of excitatory-inhibitory synaptic imbalance and MN excitability properties in ALS has not yet been established.

Altogether, these factors have been identified as key determinants that help elucidate the shared underlying mechanisms responsible for MN susceptibility to diverse pathological processes throughout ALS. This convergence of factors underscores the complex nature of the disease

1.5. Skeletal muscle involvement in ALS

Several research findings indicate that skeletal muscle can experience early and independent alterations in ALS, separate from the denervation process propelled by deteriorating MNs. Skeletal muscle repair and regeneration rely on efficient muscle satellite cells (MuSC) activation, proliferation, fusion, and differentiation, a myogenesis process perturbed in ALS even before the onset of symptoms (Tsitkanou et al., 2016). Using magnetic resonance imaging (MRI) techniques, additional studies have unveiled that muscle volume in mSOD1 mice is diminished as early as 8 weeks of age (Marcuzzo et al., 2011).

Initially, it was observed that genetically reducing mSOD1 expression in muscle did not impact disease progression or survival. Similarly, the adeno-associated viral vector (AAV)-mediated overexpression of follistatin in the muscles of mSOD1 mice stimulated muscle growth but did not influence survival (Miller et al., 2006). In a complementary approach, it was demonstrated that muscle-specific expression of mSOD1 could lead to muscle weakness, atrophy, and mitochondrial dysfunction but did not alone trigger MN degeneration (Dobrowolny et al., 2008). However, in contrast to these outcomes, subsequent studies revealed that overexpression of mSOD1 in muscle did replicate abnormalities at the NMJ, axonopathy, and MN degeneration (Wong & Martin, 2010). The muscle by itself could be involved in NMJ dismantlement by upregulating axonal outgrowth inhibitors such as Nogo-A, which correlates with the degree of denervation and symptom severity in patients (Bruneteau et al., 2015; Jokic et al., 2005). Furthermore, the skeletal muscle contributes to MN degeneration in spinal and bulbar muscle atrophy, different forms of MND (Cortes et al., 2014). Collectively, these discoveries establish the proof of concept that specific muscle abnormalities can disrupt motor nerve terminals and thereby play a role in the development of ALS.

1.6. Forward or backward motoneuron degeneration

The distinct involvement of LMNs and UMNs, and evidence outlining the mechanisms of disease progression and propagation have led to the question of whether MNs are undergoing a "dying-forward" or "dying-backward" process. Changes in skeletal muscle innervation begin before MN degeneration and were first reported in the histological analysis of a patient who died unexpectedly before reaching the end stage. Muscle wasting and denervation were noticeable in the leg and thoracic skeletal muscles, but there was no axonal degeneration within the ventral roots, and the spinal cord retained normal numbers of MNs (Fischer et al., 2004). Later studies in the SOD1^{G93A} mouse model and ALS patients have corroborated this notion (Corti et al., 2009; Krasnianski et al., 2005; Mahoney et al., 2006; Mancuso et al., 2011, 2014).

Dengler and colleagues (1990) indicated early alterations in the function of distal peripheral nerves in patients who exhibited initial muscle weakness. Moreover, electrophysiological data correlates with structural irregularities in the incoming nerve, the muscle, and TSCs in individuals at the early disease stage, indicating once more that changes at NMJ manifest early in the course of the disease (Bruneteau et al., 2015). Accumulation of p-TDP-43 aggregates is detected within the motor nerves of living ALS patients, happening before axonal degeneration, an early event that could contribute to pathogenesis (Kurashige et al., 2022; Riva et al., 2022). Consistent with these findings, neuromuscular denervation appears to occur independently from MN death (Dewil et al., 2007; Gould et al., 2006). In all, this emphasizes the idea that therapeutic approaches should target both the neuronal cell body and the NMJ milieu to maximize their positive impact.

The dying-forward hypothesis, first postulated by Eisen and colleagues (1990), indicated that cortical dysfunction is an early feature of ALS that precedes LMN involvement. Physical contacts between nerve cells along axons are thought to be determinant for the dissemination of ALS pathology. Several observations have strengthened this concept. First, extraocular and sphincter muscles have limited cortical connections. Second, ALS cases with exclusively LMN involvement are rare. Third, non-human species have underdeveloped direct corticomotor neuronal connections to spinal MNs, which could account for the lack of naturally occurring animal models of ALS (Eisen et al., 1992; Lemon & Griffiths, 2005). Fourth, the initial asymmetric and localized motor impairments seen in ALS may be linked to the intricate anatomy of the synapses between UMNs and anterior horn cells. Additionally, the asymmetry observed in the pattern of onset and spread has been attributed to limb dominance, with UMN processes playing a significant role (Devine et al., 2014).

In line with this, cortical hyperexcitability can facilitate the degeneration of MNs through an excitotoxic mechanism that operates in a forward manner, involving trans-synaptic processes. Cortical hyperexcitability could stem from the loss of inhibitory GABAergic circuits in the primary

motor cortex, leading to disinhibition, which would be exacerbated by hyperactivity of excitatory cortical interneurons (Nihei et al., 1993; Zhang et al., 2016). This is coupled with cortical neuronal degeneration, dendritic spine loss, and apical dendritic retraction (M. J. Fogarty et al., 2015; Jara et al., 2012; Saba et al., 2016). The genetic discovery of the *C9ORF72* locus hexanucleotide expansion also offered insights into the significance of cortical dysfunction in the development of ALS-FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

All these findings contribute to the expanding body of evidence supporting the concept of a "dying-forward" process in ALS. However, the notion that UMNs appear vulnerable in a dying-forward fashion, while LMNs seem to display an alternate dying-back pathology, raised a third school of thought: the independent hypothesis.

The independent degeneration hypothesis suggests that UMNs and LMNs degenerate independently and in a stochastic manner, leading to disease spread patterns that are both contiguous and random, following the underlying neuroanatomical boundaries (van den Bos et al., 2019).

1.7. ALS experimental models

1.7.1. *In vitro* models

Conducting experiments with *in vitro* models allows the study of precise cellular mechanisms and pathways and the screening for potential pharmacological targets before moving to complex animal models. A commonly utilized cell culture model in ALS involves the mouse cell line NSC-34 (Gomes et al., 2010). Following differentiation, NSC-34 cells exhibit numerous MN properties, including the ability to generate action potentials, the expression of NF proteins, and the synthesis of acetylcholine, as reported by Cashman et al. (1992). Furthermore, when NSC-34 cells are transfected with mSOD1, they exhibit the formation of cytoplasmic inclusions (Turner et al., 2005). Nonetheless, the model simplicity becomes a limitation when investigating interactions with other cell types that contribute to the non-autonomous cell death characteristic of ALS. This adds to the setback that this model is not suitable for studying glutamate-induced excitotoxicity (Madji Hounoum et al., 2016).

Primary MN cultures from the dissociated spinal cord of mouse or rat embryos represent a better model for studying neurotoxicity or neuroprotection (Nagai et al., 2007; Petrozziello et al., 2017). Primary co-cultures of different cell types can be performed to explore the contribution and interplay of glial cells in the context of ALS research (Díaz-Amarilla et al., 2011), and allow the study of MNs from postnatal and adult animals. Organotypic cultures from animal explants are a

more feasible approach to maintaining a close-to-physiological cytoarchitecture. Spinal cord organotypic cultures (SCOCs) are particularly suited for modeling glutamate toxicity by direct exposure to D-glutamate or the addition of the glutamate transport inhibitor DL-threo- β -hydroxyaspartic acid (THA) (Guzmán-Lenis et al., 2009; Rothstein et al., 1993).

This model allows testing of neurotoxic or neuroprotective effects of compounds, while partially maintaining neural circuits, cellular stoichiometry, and similar *in vivo* conditions. However, SCOCs are axotomized systems that not only lose their target innervation but also entail an inherent cell death and marked glial response. An increasingly growing approach is the use of iPSCs and organoids allowing the study of cells from both fALS and sALS patients (Burkhardt et al., 2013; Dimos et al., 2008). iPSCs are derived from patients' somatic cells, that are reprogrammed and differentiated into MNs or glial cells. iPSCs offer the advantage of having lines derived from patients harboring different mutations or from sporadic causes (Burkhardt et al., 2013; Hawrot et al., 2020; Zhao et al., 2020). Furthermore, iPSCs can be guided-differentiated into different cell types and form organoids, 3D culture systems that recreate the architecture and physiology of human organs. For instance, ALS organoids have been employed to recapitulate the mature cortical architecture (Szebényi et al., 2021) or to model functional NMJs (Pereira et al., 2021).

1.7.2. *In vivo* rodent models

The identification of numerous genes associated with ALS over the past two decades has led to the development of transgenic models intending to replicate and study the disease. An ideal rodent model for ALS should recapitulate ALS phenotypes and pathologies; additionally, it may exhibit FTD features (Fig. 3A). Mutations in the SOD1 gene on chromosome 21 were the initial genetic factors discovered to be causative of ALS (Rosen et al., 1993). Subsequent research into the aberrant function of mSOD1 has played a crucial role in advancing the comprehension of ALS pathogenesis. The first animal model featured a high copy number of the human mSOD1 gene with a glycine-to-alanine transition at the 93rd codon (Ripps et al., 1995). These mice exhibit the onset of motor symptoms around 12 weeks of age, developing tremors and weakness, which is preceded by axonal degeneration and NMJ denervation. Gradual and rapid degeneration of spinal MNs and an increase in reactive glia results in fatal hindlimb paralysis, leading to death at approximately 4-5 months of age (Fig. 3B). Importantly, this model is still the gold standard in ALS research since it replicates most clinical and histopathological characteristics seen in the most common clinical profile of ALS with predominant involvement of spinal MNs, of both familial and sporadic causes, except for the mislocalization and accumulation of TDP-43 inclusions and FTD (Mancuso & Navarro, 2015).

Several models bearing other described mutations in the SOD1 gene have been generated, such as the SOD1^{G37R} or the SOD1^{G85R}, or models carrying a low copy number of the SOD1^{G93A}. Typically, these mice exhibit a later onset and a slower disease progression. Additionally, transgenic rat models featuring SOD1 mutations mirroring the disease phenotype were developed (Nagai et al., 2001), although they show more variability in the disease course.

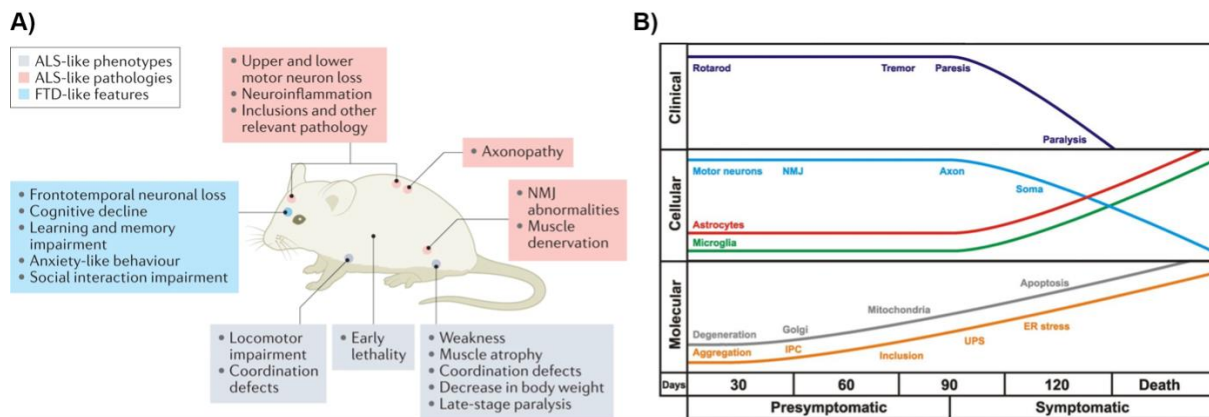


Figure 3. ALS mouse models. (A) ALS-like phenotypes, pathologies, and FTD features observed in mouse models of ALS (extracted from Todd & Petrucelli, 2022). (B) Time course of clinical and neuropathological events in high copy number transgenic SOD1^{G93A} mice (from Turner & Talbot, 2008).

Models for *TARDBP* mutations have been generated with the wild-type or mutant *TARDBP* sequences with ranging degrees of MN degeneration and phenotypes, regrettably, these models failed in reproducing a typical ALS-like phenotype (Huang et al., 2020; Watanabe et al., 2020). Homozygous loss of *TARDBP* is embryonically lethal, whereas heterozygous loss results in muscle weakness but no MN loss (Kraemer et al., 2010). The initial Prp-TDP-43 mouse models exhibited motor deficits and frontotemporal lobar degeneration. Nuclear TDP-43 was lost in some neurons but was not detected as cytoplasmic aggregates (Stallings et al., 2010; Wils et al., 2010; Y.-F. Xu et al., 2010). To overcome this problem, several groups introduced mutations into the nuclear localization sequence (NLS), generating models that display ALS-like phenotypes (Sasaguri et al., 2016; Walker et al., 2015).

FUS may induce toxicity through comparable mechanisms to TDP-43 (Ratti & Buratti, 2016). However, individuals with ALS carrying FUS mutations develop FUS-positive, but not TDP43-positive inclusions. Like TDP43 models, FUS rodent models can be categorized broadly into animals overexpressing WT FUS, *Fus*-knockout animals, and mutant models. In contrast, murine models with *Fus* mutations display ALS-like locomotor deficits and a progressive degeneration of MNs (López-Erauskin et al., 2018; Zhang et al., 2020).

C9ORF72 hexanucleotide repeat expansion is the most common alteration found in ALS patients. The G4C2 repeat expansion is believed to induce toxicity through two gain-of-function mechanisms, leading researchers to generate inducible and bacterial artificial chromosome (BAC)

transgenic C9FTD/ALS models, each with varying numbers of expansion repeats and distinct phenotypes. Some of these models do not exhibit TDP-43 pathology, while others lack some of the five DPRs or fail to induce MN loss (Y. Liu et al., 2016; O'Rourke et al., 2015; Riemsdijk et al., 2021). Thus far, ALS features have been best recapitulated through AAV-mediated somatogenesis by introducing G4C2 repeats into the mouse CNS (J. Chew et al., 2015, 2019).

Overall, the phenotypes associated with TDP43, C9ORF72, or FUS models appear less robust than those observed in mSOD1 transgenic animals and may only become apparent at an advanced age.

Of note, mutations in the gene encoding profilin 1, a ubiquitous cytosolic protein that participates in the assembly of filamentous actin (F-actin), have been identified in several familial ALS cases. The mouse line hPFN1^{G118V} expressing high levels of mutant human profilin 1 exhibited many clinical and pathological features like human ALS. These include loss of lower and upper MNs, abnormally ubiquitinated proteins, reduced ChAT enzyme expression, glial cell activation, axonal degeneration in ventral roots, muscle atrophy, impaired motor performance, and reduced survival (Fil et al., 2017).

1.8. Available therapeutic strategies for ALS

Overall, multidisciplinary care, symptom control, and palliative measures, including nutritional, physical, and respiratory support, are the cornerstones of patients' management. Unfortunately, to date, there is no effective treatment for ALS. Despite decades of intense research and clinical trials, most promising preclinical therapies have failed to translate into successful human trials. riluzole, edaravone (RadicavaTM), Relyvrio (AMX0035), and Qalsody (Tofersen) are the only treatments in the market for ALS. The first medication approved was riluzole, its positive effect was attributed to its anti-glutamatergic properties, which involve the inhibition of NMDA receptors and voltage-dependent sodium channels (Doble, 1996). However, despite its widespread use, riluzole offers only a modest benefit in survival with an average life extension of 3-4 months (Bensimon et al., 1994).

Edaravone demonstrated beneficial effects in a phase III clinical trial involving early-stage ALS patients (Abe et al., 2017). The exact way edaravone works in patients is not known but it is thought to be a scavenger of peroxy radicals and peroxynitrite, primarily exerting its effect by mitigating oxidative stress. Notably, clinical trials with this medication exhibited efficacy in a limited subset of individuals with ALS who met specific criteria in a post-hoc analysis (Edaravone ALS Study Group, 2017). This medication was withdrawn in Europe as the European Medicines Agency

(EMA) concluded there was no positive benefit-risk balance. Relyvrio was approved, a few years ago, in the United States. Relyvrio is a combination of two drugs, sodium phenylbutyrate and taurursodiol. This combination slows disease progression by preventing MN death by blocking stress signals from the ER and the mitochondria. Clinical trials have suggested that Relyvrio has both modest functional and survival benefits for ALS (Paganoni et al., 2020, 2021).

In 2023, Qalsody or Tofersen, an investigational drug, received approval in the United States for the treatment of SOD1-ALS under the accelerator approval pathway. Tofersen is an antisense oligonucleotide (ASO) administered intrathecally and designed to specifically target *SOD1* mRNA. The initial readouts of this clinical trial indicate that Tofersen lowered the levels of SOD1 protein in the CSF and reduced the bloodstream concentration of NFL. Although it did not meet its primary endpoint of slowing disease progression, there were some trends towards benefit. A follow-up study showed that earlier initiation slowed the decline in strength, clinical and respiratory function, and quality of life (Miller et al., 2022).

Interestingly, most of the compounds showing beneficial effects in preclinical studies and that failed in clinical trials interfere with a single mechanism involved in MN death (Benatar, 2007). One of the main difficulties in developing effective therapies for ALS arises from the multiple events that collectively contribute to the etiopathogenesis of MN death.

2. Gene therapy

2.1. Gene therapy overview

Gene therapy is commonly described as a therapeutic approach involving the introduction of DNA/RNA into an individual's body to modify a malfunctioning gene and alleviate a disease that is unresponsive to traditional pharmacological treatments. Gene therapy strategies can be categorized based on several factors; the type of disease (genetic disorder or complex acquired condition), the gene delivery system (integrating or nonintegrating), and whether the vector is applied *in vivo* (directly into the patient) or *ex vivo* (by modifying autologous cells in culture and then retransplanting) (Kumar et al., 2016). The basic concept of gene therapy involves the identification of a missing gene in a genetic disorder, integrating the gene into a vector, and delivering it to the affected patients. (Theodore Friedmann, 1972). Consequently, transduced cells will possess in *trans* a copy of the absent gene, leading to the resolution of the disease (Fig. 4A). The growing interest in gene therapy was incited by the realization that a single treatment could provide lasting and potentially curative clinical benefits. Unlike protein-based drugs, which necessitate repeated administrations, gene-based therapies introduced into long-lived cells could

offer sustained production of natural proteins. Initially conceived primarily for inherited or severely disabling disorders, gene therapy is now being extended to acquired conditions (Dunbar et al., 2018; Kumar et al., 2016; Mansilla-Soto et al., 2016).

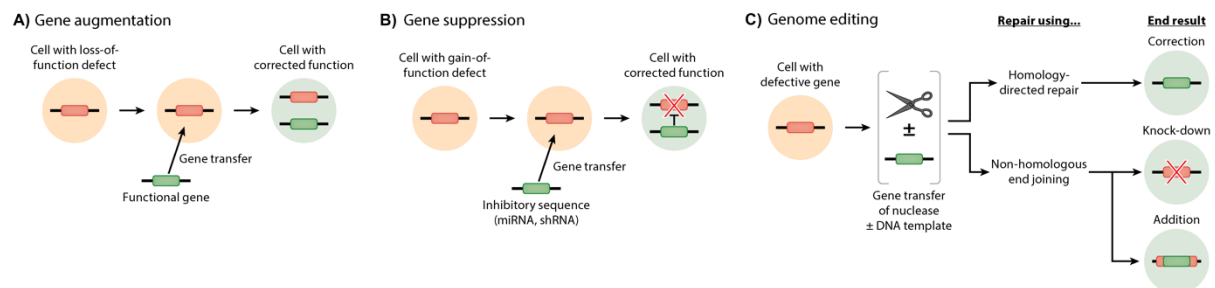


Figure 4. Gene therapy fundamentals. Gene therapy encompasses gene augmentation strategies to supply therapeutic or missing genes (A), and gene suppression (B) or genome editing (C) strategies to correct faulty genes (From Anguela & High, 2019).

In some conditions, the loss of cellular function stems from the harmful accumulation of a faulty protein or RNA. One can inhibit the expression of this harmful gene by utilizing RNA interference or genome editing tools (Fig. 4B). Genome editing can be employed to rectify a mutated gene at its specific genomic position by inducing a DNA double-strand break. The choice between different DNA repair mechanisms will determine the outcome of genome editing (Fig. 4C) (Anguela & High, 2019).

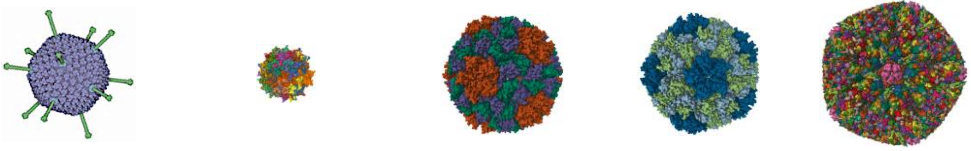
Various gene therapy vectors have been created to transport therapeutic nucleic acids into cells, categorizing them into two main strategies: non-viral and viral vectors. Most gene therapy strategies are currently converging on viral vectors: lentiviral vectors for *ex vivo* gene delivery into hematopoietic and other stem cells, and adeno-associated viral (AAV) vectors for *in vivo* gene transfer into postmitotic cell types.

When introducing genetic material into stem cells or dividing cells, it is of utmost importance to employ integrating vectors, ensuring that upon cell division the introduced DNA is inherited by descendant cells. In the context of *in vivo* gene therapy, the focus often shifts towards long-lived postmitotic cells. In these cells, which no longer undergo division, achieving long-term expression becomes feasible as long as the transferred DNA remains stable within the cell. Episomal stabilization is sufficient to sustain expression throughout the cell's lifespan (Anguela & High, 2019; Naldini, 2015). Currently, almost 1,200 clinical trials are being assessed on gene therapy (ClinicalTrials.org). While global approvals and market availability exist for only 19 gene therapies, primarily for rare diseases, a significant number of patients have received these treatments. This has demonstrated the feasibility and safety of gene therapy when administered at appropriate dosages. For instance, in the case of spinal muscular atrophy (SMA) type I, to date over 3,000 children have undergone treatment with Zolgensma (onasemnogene abeparvovec).

2.2. Viral vectors for gene therapy

The key step in gene therapy involves the effective transport of genes to the intended tissues or cells, a task accomplished by gene delivery vectors. Over millions of years, viruses have developed remarkable efficiency in bypassing the organism's defenses, infiltrating tissues, and infecting cells to introduce their genetic material. This inherent capability has granted them a superior gene delivery potential compared to non-viral vectors. As a result, various types of viral vectors, originating from both DNA and RNA viruses, have been deliberately engineered for applications in gene therapy.

Table 1. Types of viral vectors and their characteristics. (Adapted from Lundstrom, 2023. *Capsid images retrieved from Protein Data Bank*).



Viral vector	Adenovirus (Ad)	Adeno-associated virus (AAV)	Retrovirus (RV)	Lentivirus (LV)	Herpesvirus (HSV)
Size (nm)	80	25	100	80-100	150
Genome	dsDNA	ssDNA	ssRNA	ssRNA	dsDNA
Packaging capacity	4.5-36 Kb	5 Kb	8 Kb	9 Kb	30-150 Kb
Tropism	Broad (low for blood cells)	Broad (depending on capsid)	Dividing cells	Broad (low for blood cells)	Broad
Transgene expression	Transient (persistent for gutless Ad)	Transient or stable	Stable	Stable	Transient
Cell transduction	High. Episomal	Moderate. Episomal (90%)	Moderate. Integrative	Moderate. Integrative	Moderate. Episomal
Immune response	Strong	Weak	Moderate	Moderate	Strong
Advantages	Large packaging capacity and high transduction efficiency. Excellent as oncolytic vectors.	Least immunogenic vectors. Safe transgene delivery.	Persistent gene transfer. Producer cell lines for large-scale manufacturing.	Persistent gene transfer. Broad host range. Producer cell lines for manufacturing. Preferred for ex vivo applications.	Broad host range (strong in neurons with retrograde transport). Large packaging capacity. Helper-free packaging system.
Limitations	Potent immunogenicity and pre-existing immunity.	Limited packaging capacity. Strong immune response in re-administration. Low in vitro infectivity.	Restricted to dividing cells. Random integration. Insertional mutagenesis.	Integration selectivity. Insertional mutagenesis.	Cytotoxicity and pro-inflammatory responses.

The selection of a specific vector is significantly influenced by the nature of the disease being targeted and the necessity for either short- or long-term expression (Bulcha et al., 2021; Lundstrom, 2023). A comprehensive overview of the most common viral vector types and their properties is provided above (Table 1). In any gene therapy application based on viral vectors, safety is of the greatest importance. The long-term treatment and the presence of viral vector and/or transgene sequences within the host's genome necessitate specific considerations. These include insertional genotoxicity, immune-mediated destruction of modified cells, immune reactions linked to the administration of vectors, and control of the expression levels and pharmacokinetics of the therapeutic product (Lundstrom, 2023).

2.3. Adeno-associated viral vectors (AAV)

2.3.1. AAV biology

Adeno-associated viruses (AAV) are members of the *Parvoviridae* family and the Dependoparvovirus genus. These viruses are replicant-deficient and rely on the presence of a helper virus, such as adenovirus (AdV), to complete their life cycle. AAV consists of an icosahedral protein capsid housing a single-stranded (ss) DNA genome, approximately 4.7 kb in size, which can exist as either a sense (plus) or anti-sense (minus) strand. The AAV genome is bookended by two T-shaped palindromic inverted terminal repeats (ITRs) at its ends, which function as origins of replication and packaging signals. The capsids are composed of three capsid protein subunits — VP1, VP2, and VP3— in a ratio of 1:1:10 (VP1:VP2:VP3), resulting in a total of 60 copies. The *Cap* gene encodes the three capsid subunits through alternative splicing and translation from different start codons. For viral replication, four *Rep* genes are essential, encoding four proteins: Rep 78, Rep 68, Rep 52, and Rep 40. The assembly activating protein (AAP), plays a crucial role in facilitating virion assembly (Wang et al., 2019). While, the membrane-associated accessory protein (MAAP), also expressed from a frameshifted reading frame in the cap gene, has recently been described to be a viral egress factor (Elmore et al., 2021).

The transcription of these genes is driven by the viral P5, P19, and P40 promoters (Fig. 5A). The assembled capsid exhibits distinct characteristics, with the most prominent feature being the five-fold axis of symmetry. This five-fold axis serves as the binding site for rep proteins and encompasses the central pore, where DNA is introduced through rep-mediated ATPase activity. Additionally, there is the threefold axis of symmetry, which is defined by the presence of threefold protrusions composed of variable loop regions V, VII, VIII, and IV. These variable loops play a pivotal role in establishing the primary surface epitope for both antibody recognition and receptor binding (Fig. 5C, D).

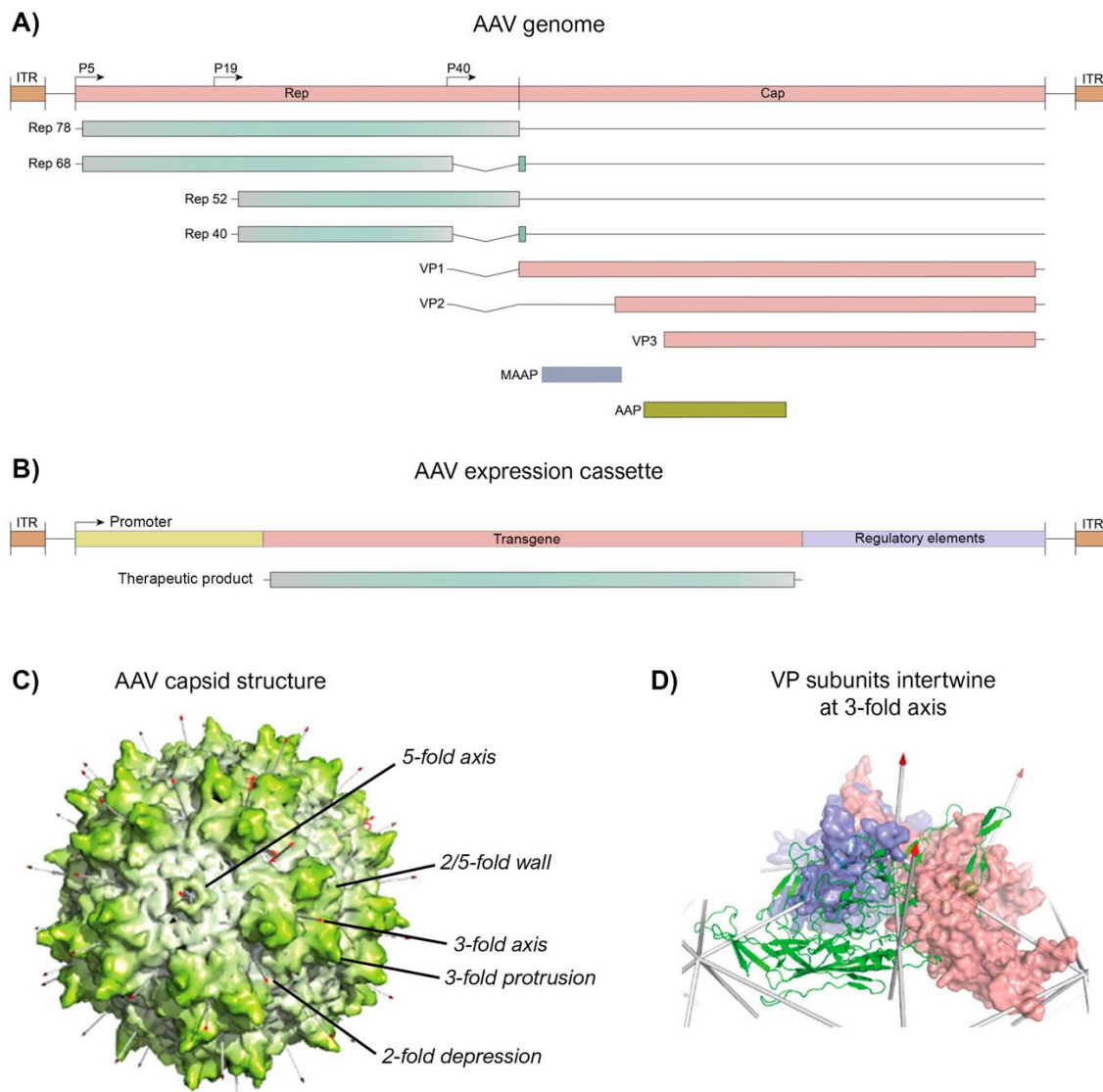


Figure 5. AAV genome and capsid structure. (A) The AAV genome has four known open reading frames, rep (green), cap (salmon), MAAP (orange), and AAP (yellow), flanked by ITR sequences. (B) The AAV genome is gutted and replaced by an expression cassette in AAV viral vectors. (C) The capsid of AAV is viewed approximately down a 5-fold axis, which is composed of 60 copies of a combination of VP1, VP2, and VP3 that intertwine (D). (Adapted from Stagg et al., 2022; Wang et al., 2019).

Over a thousand AAV variants have been described across the five primate clades. Each serotype is thought to exhibit distinct binding characteristics, with varying affinities for primary cell surface glycoprotein receptors as well as secondary receptors that allow entrance into target cells (Mietzsch et al., 2014). The most common AAV vector serotypes used in preclinical and clinical contexts are summarized in Table 2.

Following binding to the cell surface, the AAV particle is clathrin-mediated endocytosed and undergoes vesicular trafficking, progressing through late endosomal and lysosomal compartments (Fig. 6). In these vesicles with low pH environments, the capsid goes through a conformational change, exposing the VP1/2 domains (Sonntag et al., 2006).

Table 2. Common serotypes/variants for AAV vectors (Adapted from Pupo et al., 2022).

AAV Serotype	Origin	Tissue tropism	Primary Receptor	Secondary Receptor(s)
AAV1	Primate	Heart, Muscles, Liver	N-linked sialic acid	AAVR, GPR109, TM9SF2
AAV2	Human	Liver, Eye, Muscle	HSPG	AAVR, GPR108, TM9SF2, LamR, $\alpha\beta 5$ integrin, $\alpha\beta 1$ integrin, FGFR1, CD9, HGFR
AAV3	Human	Liver, Heart, Muscle	HSPG	AAVR, GPR108, LamR, FGFR1 c-MET, HGFR
AAV4	NHP	Liver, Kidney	O-linked sialic acid	GPR108
AAV5	Human	Liver, Muscle	N-linked sialic acid	AAVR, PDGFR, TM9SF2
AAV6	Human	Lung, Liver, Muscle	HSPG, N-linked sialic acid	AAVR, GPR108, TM9SF2, EGFR
AAV7	Rhesus macaque		-	GPR108, TM9SF2
AAV8	Rhesus macaque	Liver, Muscle	-	AAVR, PDGFR, TM9SF2, LamR
AAVrh.8	Rhesus macaque		-	GPR108
AAV9	Human	Heart, Muscle, CNS, Liver	Terminal N-linked galactose of SIA	AAVR, GPR108, TM9SF2, LamR
AAV10	Cynomolgus monkeys		-	-
AAVrh10	Rhesus macaque	Brain, Muscle, Liver	-	GPR108, LamR
AAV11	Cynomolgus monkeys	SM, kidney, spleen, lung, heart, stomach	-	-
AAV12	NHP	SM, salivary glands	-	-
AAV-DJ	Synthetic chimeric	Broad tropism	HSPG	-

The VP1-unique region, housing a phospholipase domain, escapes the endosome or lysosome through a mechanism not yet fully elucidated. The particle is then directed into the nucleus via nuclear localization signals present on VP2. Once inside the nucleus, the AAV uncoats and the genome undergoes second-strand synthesis to have the double-stranded configuration necessary for gene transcription. The ITRs facilitate recombinations giving rise to circular double-stranded DNA species or concatenated genomes. This process ensures the stability of the AAV genome in an episomal state (D. Duan et al., 1998). Notably, a low proportion (approximately 10%) of the wild-type AAV can integrate into the host cell's genome. The most common and well-known integration site for wild-type AAVs in the human genome is a locus on chromosome 19 (AAVS1) because of existing homology with the ITRs, albeit this integration is not linked to insertional mutagenesis (Kotin et al., 1991).

2.3.2. Recombinant AAV viral vectors and expression cassettes

Samulski et al. (1982) were the pioneers in cloning the AAV genome into expression plasmids. Their groundbreaking work demonstrated that when these plasmids were transfected into mammalian cell lines in the presence of AdV, they could generate infectious viruses. Subsequent advancements in recombinant AAV (rAAV) technology revealed that it was possible to package

transgenes by essentially emptying the genome, except for the ITR elements, and replacing it with any desired gene and regulatory sequences (Fig. 5B). Once the viral genome coding for the transgene is within the nucleus of the host target cell and in the episomal state, transcription will start according to the exogenous promoter, generating the desired proteins or RNAs (Fig. 6 [7]).

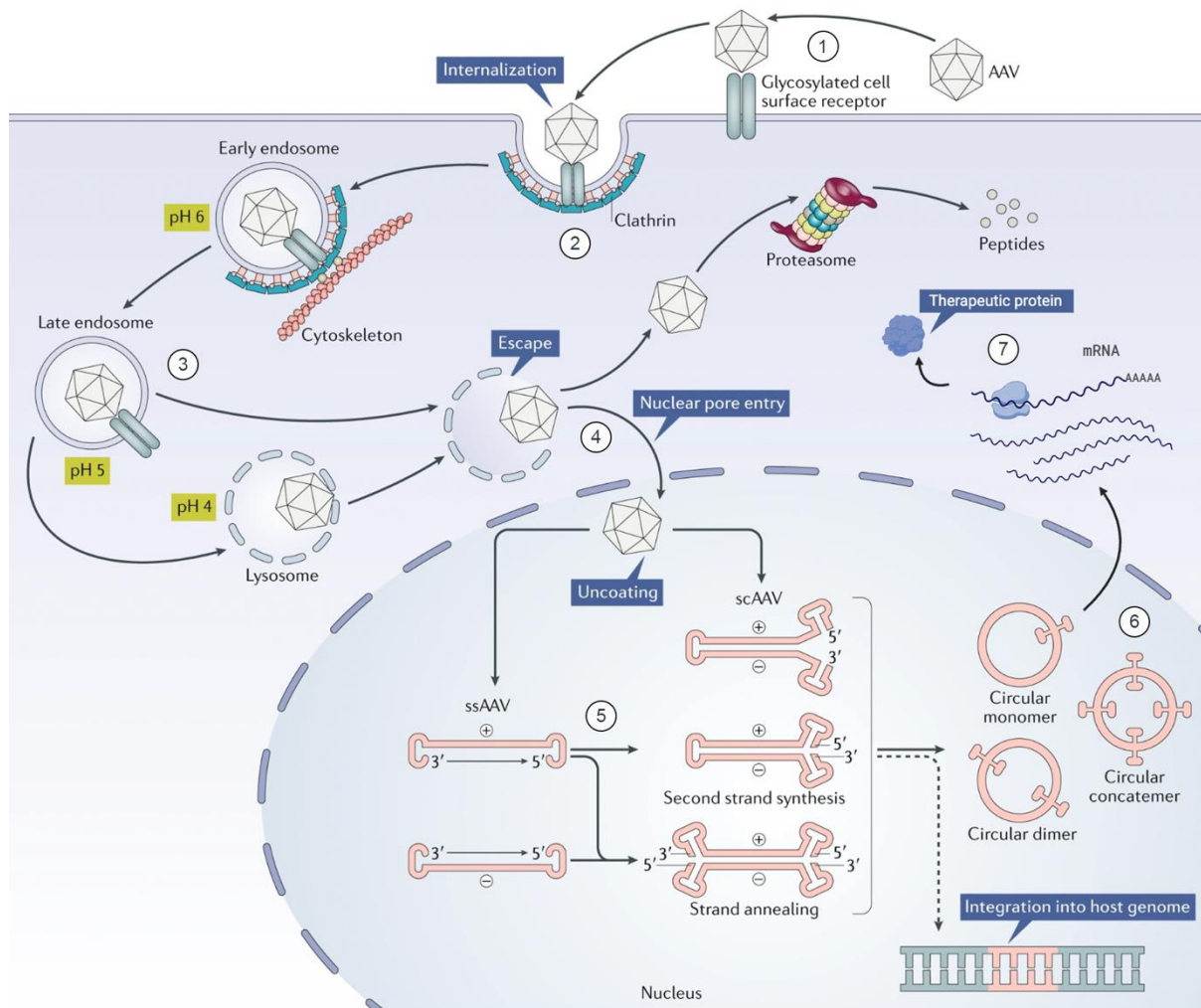


Figure 6. rAAV transduction pathway. (1) AAV binds to the cell surface, is endocytosed (2), and progresses through late endosomal and lysosomal compartments (3), from where the particle escapes (4). The virion is directed to the nucleus, where the genome undergoes second-strand synthesis (5) and forms circular double or concatenated genomes (6). The viral genome can now be transcribed and translated to proteins (7). (*Adapted from Wang et al., 2019*).

The process of crafting these vectors requires an ingenious endeavor driven by multiple factors. The primary consideration in AAV vector design is that the natural genome is approximately 4.7 kb in size. Consequently, AAV vectors are inherently restricted to a maximum capacity of 5 kb. Strategies leveraging ITR-mediated recombination have resulted in dual-vector systems capable of expressing "oversized" transgenes through transcript splicing across two complementary vector genomes (Duan et al., 2000; Nakai et al., 2000). Other methods to expand vector size through homology-based vector recombination (Ghosh et al., 2008), RNA trans-splicing (Lai et al., 2005), or split intein designs for protein "trans-splicing" (W. L. Chew et al., 2016) have also been exploited.

The selection of promoters for driving transgene expression in AAV vectors is another crucial facet of vector design. Given that AAV serotypes or engineered capsids can transduce various tissue and cell types, achieving cell-type-specific transgene expression primarily relies on controlling gene transcription. The common approach is the use of tissue-specific promoters. Importantly, these regulatory cassettes must not only be tissue-specific but also possess the strength necessary to confer therapeutic levels of transgene expression. However, recent studies have shown that, in monogenic disorders, achieving physiological expression levels of therapeutic products is more desirable to avoid toxic effects due to too high or too long-term levels (Van Alstyne et al., 2021). The activation of promoters can also be finely controlled temporally using inducible promoters, which are activated only when exposed to a particular molecule that is administered to the organism.

rAAVs can be generated in laboratory and clinical settings using HEK293 adherent cells and the triple transfection method, where cells are simultaneously transfected with three plasmids. One plasmid supplies the necessary helper genes, mimicking the role of the helper virus in a natural environment. The second plasmid contains the AAV rep2 and cap genes along with the specific capsid serotype sequence. Lastly, the third plasmid harbors the gene therapy expression cassette flanked by the ITR sequences (Wang et al., 2019). For large-scale clinical-grade vectors, HEK293 cells have been adapted to grow in suspension in bioreactors (Grieger et al., 2016).

Last, capsid engineering methods to find novel serotypes have been a constant pursuit since the initial recognition of the clinical potential in rAAVs. Approaches to capsid development can be categorized into four primary groups: natural discovery, rational design, directed evolution, and *in silico* discovery. Exploring naturally occurring AAV involves studying proviral sequences found in host tissues that might have experienced wild-type AAV infections. Directed evolution employs methods such as capsid shuffling (chimeric natural serotypes) or error-prone PCR to generate huge libraries of distinct capsid combinations with unique vector properties. Rational design leverages existing knowledge of AAV biology and host cell targets to engineer capsids capable of recognizing a receptor in a specific cell or tissue.

On the other hand, *in silico* design employs computational methodologies to forecast novel capsid designs not found in nature. The identified candidate capsids need to be screened and assessed for their potential in *in vitro* systems and animal models (Wang et al., 2019). As an example, the novel MyoAAV capsid has been engineered through directed evolution techniques (Tabebordbar et al., 2021). This capsid exhibits an improved affinity for striated muscle, resulting in potent transduction of muscles throughout the entire body and outperforming naturally occurring AAVs.

2.4. Gene therapy in ALS

Gene therapies offer significant potential in addressing rare, devastating, and life-threatening diseases like ALS, as they usually focus on correcting the root cause of the condition rather than just treating the symptoms. The notable success in treating infants with SMA type I in recent years underscores the promise of gene therapy for other neuromuscular disorders and MNDs. However, unlike SMA type I, ALS is uncommonly a monogenic disorder, making therapeutic strategies less straightforward. Additionally, in cases where there is a genetic basis, the disease is mostly associated with gain-of-function mutations, rendering gene replacement therapies impractical.

Numerous strategies in gene therapy have been conducted in preclinical settings, primarily aiming for gene augmentation or the overexpression of factors capable of mitigating pathological alterations associated with ALS (outlined in Appendix Table 11). The direct suppression of disease-causing genes through ASOs or by viral-mediated knockdown with short hairpin RNAs (shRNAs) or miRNAs has been extensively explored (summarized in Appendix Table 12) and is showing promise in clinical trials.

2.4.1. Gene delivery

The delivery of exogenous genes and their supraphysiological expression has primarily centered around enhancing the presence of neuronal prosurvival and trophic factors, as well as modulators of neuromuscular junctions. On the other hand, alternative strategies aiming to prevent apoptosis, impede protein aggregation, or promote glutamate clearance have been studied to mitigate disease progression and selective MN degeneration.

Neurotrophic factors (NTFs) support the growth, survival, synaptic plasticity, and differentiation of neurons. NTFs have demonstrated the ability to regenerate damaged neurons in vitro and animal models. Glial cell line-derived neurotrophic factor (GDNF) is one of the strongest neurotrophins and its delivery through different routes and with different targets has been experimentally assessed in many studies. Overexpression in the spinal cord has been tested in SOD1^{G9A3} murine models, (Guillot et al., 2004; Klein et al., 2005; Thomsen et al., 2017). However, despite observing a robust GDNF expression, the resulting MN preservation and motor improvements were minimal to negligible. To induce axon guidance cues and GDNF retrograde transport, several groups opted for intramuscular (i.m.) injections of AAVs, Ad, or ex vivo-modified mesenchymal stem cells (MSCs). When administered into a single muscle, no clinically significant benefits were observed, despite the preservation of MNs innervating that muscle (Manabe et al., 2002).

On the contrary, we and others demonstrated that simultaneous administration into various muscles delayed disease, protected MNs, preserved NMJs, delayed onset, and prolonged survival (Acsadi et al., 2002; Lu et al., 2003; Mòdol-Caballero et al., 2021a; Mohajeri et al., 1999; Suzuki et al., 2008).

In analogous methods, insulin-like growth factor 1 (IGF-1) was evaluated through i.m. injections into various muscles of SOD1^{G93A} rodents using AAV or LV vectors. IGF-1 overexpression helped in the preservation of MNs, enhanced gait and rotarod test performance, and prolonged survival (Eleftheriadou et al., 2016; Kaspar et al., 2003; Lin et al., 2018; Wen et al., 2019). When focusing on MNs, i.s. injections exclusively maintained MNs within treated segments (Franz et al., 2009). The protective effects of IGF factors have been postulated to be partly dependent on the vascular endothelial growth factor (VEGF). A study reported an extension of lifespan and a delay in the disease onset after overexpression of VEGF in various muscles of SOD1^{G93A} mice (Azzouz et al., 2004). In a different approach, i.t. administration of scAAV9-VEGF also fostered survival and motor performance (Wang et al., 2016), but to a much lesser extent. Other NFs have been examined using viral methods, but these have displayed less favorable outcomes (Bordet et al., 2001; Haase et al., 1998; Henriques et al., 2011; Lee et al., 2019a; Lee et al., 2019b).

Modulators of the neuromuscular junctions have been explored as potential gene therapy options for ALS. In previous studies, we have overexpressed Neuregulin 1 type I (Nrg1-I) in the gastrocnemius muscles (GM), increasing collateral sprouting and compound muscle action potential (CMAP) amplitudes of the muscle, without protecting axons or MNs (Mancuso et al., 2016). Only when we globally overexpressed Nrg1-I in all muscles, MNs, neuromuscular and locomotor functions were preserved, delaying disease onset (Mòdol-Caballero, et al., 2020b). In an alternative strategy, DOK7, essential for NMJ, was ubiquitously expressed and did not avoid MN loss, but protected from nerve terminal and muscle atrophy (Miyoshi et al., 2017).

Other researchers have sought to prevent MN death rather than actively promoting their survival. (Azzouz, 2000) exemplified this approach by delivering Bcl-2 to the lumbar spinal cord of SOD1^{G93A} mice, preserving MNs and neuromuscular function. Still, local Bcl-2 expression was insufficient to prolong survival. Two studies have explored targeting toxic protein aggregation in the spinal cord of SOD1^{G93A} mice, one using a cytokine with chaperone-like properties (Leyton-Jaimes et al., 2019), and the other employed single-chain fragment variable antibodies (Patel et al., 2014). Both strategies effectively neutralized mSOD1 accumulation and yielded a significant postponed disease onset and survival extension. Factors such as DAO, EAAT2, or ADAR2, which have direct effects on glutamate excitotoxicity, have also been overexpressed in the CNS of ALS mice (Li et al., 2015; Wang et al., 2017; Yamashita et al., 2013). None of these therapies provided a significant improvement in strength, locomotion, or survival.

Researchers have also pursued multifactorial gene therapies for ALS, aiming for a more effective and holistic approach. The combination of LV-modified muscle progenitor cells (MPC) populations expressing different NTFs demonstrated the preservation of MNs and NMJ, delaying motor decline and extending survival (Dadon-Nachum et al., 2015; Krakora et al., 2013). Using a less conventional strategy, Benkler and co-authors (2016) delivered a multifactorial cocktail to the CNS and to the GM muscles to address the excito-oxidative axis. The mixture contained LV vectors carrying *EAAT2*, *GDH2*, and *NRF2* genes. This combination produced notably remarkable outcomes, as all three genes together but not separately improved motor parameters and survival in ALS mice.

Collectively, gene augmentation therapies focused on skeletal muscles appear to provide more favorable outcomes in MN survival, motor function, and life extension than CNS-directed strategies. Furthermore, most of the strategies offered cumulative effects when the treatments were combined, underscoring the significance of synergistic targeting in multifaceted disorders like ALS.

2.4.2. Gene silencing

SOD1 silencing

A rational approach for treating ALS involves silencing genes that contribute to the disease through a gain-of-function mutation, particularly those responsible for familial forms of ALS. The first studies delivered RNAi therapy against human *SOD1* (hSOD1) in *SOD1^{G93A}* mice using LV vectors. The study with LV vectors with more favorable outcomes targeted multiple muscles at a postnatal age and reached as much as a 77% survival increment (Ralph et al., 2005a). Another study focused on bilateral intraspinal (i.s.) injections to adult mice, substantially retarding onset and slowing disease progression (Raoul et al., 2005). As the injection into a single or few spots can only cover a small fraction of MNs, a different study directly infused modified small interfering RNAs (siRNAs) intrathecally at the symptom's onset. siRNAs efficiently knocked down hSOD1, slowed down the progression, and slightly extended survival (Wang et al., 2008b).

Intravenous administration of AAV9-shSOD1 improved motor function, retained muscle mass, and significantly extended survival in *SOD1^{G93A}* mice. Interestingly, only mice treated at P1 exhibited delayed disease onset due to more effective MN SOD1 silencing, while those treated at P21 displayed slower disease progression, linked to enhanced transduction of non-neuronal cells (Foust et al., 2013). Finally, among all SOD1 silencing strategies in the literature, the novel subpial injection of AAV9-shSOD1 has shown the most promising outcomes, successfully suppressing MND with a full correction of survival (Bravo-Hernandez et al., 2020).

MicroRNAs are part of the cell's natural regulatory machinery and rather than completely silencing can partly reduce mRNA levels. In comparison to shRNA, they offer more precise targeting, endogenous regulation, lower immunogenicity, and can be combined with specific promoters (Lam et al., 2015). Intra-CSF AAV-miRSOD1 delivery only sustains survival when administered intracerebroventricular (i.c.v.) at postnatal ages (Dirren et al., 2015; Stoica et al., 2016), intrathecal (i.t.) injections to adult mice induce SOD1 silencing and promote locomotor function but offer scarce to no correction of lifespan (Borel et al., 2018; Li et al., 2017)

ASOs have been thoroughly tested in neurodegenerative disease models and clinical trials. They are usually delivered without vectors, have a better safety profile, and ease of delivery, and provide a higher tissue penetration and rapid onset of action. The first study used modified ASO-hSOD1 continuously infused intraventricularly in SOD1^{G93A} rats and demonstrated its applicability through this route to reduce spinal cord and brain mRNA and protein levels. The treatment was initiated near onset and did not delay it, but significantly slowed disease progression and lightly extended survival (Smith et al., 2006). This led to the first-in-human phase I clinical trial of ASO delivered i.t. (Miller et al., 2013; NCT01041222). Recently, the phase III clinical trial was concluded, showcasing both reduced SOD1 CSF concentrations and NFL plasma levels. Despite not meeting the primary clinical endpoints, this ASO therapy has been approved as an investigational drug known as Qalsody (Miller et al., 2022).

The emerging field of genome editing has also been explored in the context of ALS. CRISPR-mediated editing of hSOD1 by delivery of AAV-SaCas9-sgRNA newborn pups or adult mice (W. Duan et al., 2020). Recently, gene editing using the BBB penetrating AAV-PHP.B and PHP.eB vectors in adult SOD1^{G93A} mice of AAV-PHP.eB immediately before symptoms onset fully preserved neuromuscular and motor function, and corrected survival.

C9ORF72 silencing

Silencing of C9ORF72-linked ALS has been more challenging compared to long-studied SOD1-related ALS. The genetic alteration involved in the *C9ORF72* repeat expansion is notably longer and more complex than the point mutations observed in SOD1 ALS. Furthermore, C9ORF72 ALS exhibits a high degree of genetic and phenotypic heterogeneity, complicating the development of a one-size-fits-all silencing therapy (J. Hu et al., 2015). The first study was able to demonstrate that for reducing nuclear RNA foci, ASOs must target antisense strand repeat-containing RNAs. In contrast, siRNAs failed to reduce nuclear RNA foci despite markedly reducing overall *C9ORF72* RNAs (Lagier-Tourenne et al., 2013).

In C9ORF72 BAC mice, an experimental stereopure ASO (WVE-004) showed potential by selectively and dose-dependently reducing transcripts that contained the repeat expansion (Y. Liu et al., 2022). WVE-004 is currently undergoing evaluation in a clinical trial for ALS/FTD patients possessing the G4C2-C9ORF72 expansion (ClinicalTrials.gov: NCT04931862). As for the application of genome editing techniques to block defective C9ORF72 expression, it has been examined in a limited number of studies, specifically focusing on iPSC-derived neurons derived from patients (Ababneh et al., 2020; Krishnan et al., 2020).

FUS silencing

In an initial proof-of-concept study, the *Fus* gene was initially suppressed in the common marmoset by delivering shRNA through AAV9, directly injected into the frontal cortex. This approach resulted in a significant silencing of FUS (approximately 70-80%) (Endo et al., 2018). However, no further shRNA-based approaches have been published to date. Instead, ASOs against *Fus* have been tested i.c.v. in newborn *Fus* knock-in MN-P517L/Δ14 mice, demonstrating protective effects against MN loss and denervation for up to 4 months. As ASO levels gradually decreased, microgliosis intensified, but without subsequent MN degeneration. This ASO (ION363) obtained FDA approval for experimental compassionate use. Administered i.t. and initiated 6 months post clinical onset, the ALS-FRS-R score decline rate slowed during the treatment course (Korobeynikov et al., 2022). The therapy is presently undergoing recruitment in a phase I-III clinical trial (ClinicalTrials.gov: NCT04768972).

TDP-43 silencing

TDP-43 aggregation is a component of nearly all ALS cases, and effective therapies directly targeting TDP-43 for ALS/FTD and related TDP-43 proteinopathies are lacking. Silencing TDP-43 itself may not be suitable, given its crucial cellular functions. In a 2017 study, Becker et al. proposed an alternative approach targeting ataxin-2 (*ATXN2*) to disrupt TDP-43 aggregation. Newborn TDP-43Tg/Tg mouse pups were treated i.c.v. with ASOs against *ATXN2*, resulting in a 35% extended lifespan in this severely affected model, which reaches the humane euthanasia endpoint around the 21st day of life. These data supported the advancement of this ASO (BIIB105) into a phase I/II clinical trial that is now under recruitment status (ClinicalTrials.gov: NCT04494256). In a more recent and audacious report, researchers directly induced a temporary and specific reduction of hTDP-43 in mice using 2'-O,4'-C-ethylene nucleic acids (ENAs)-modified ASO. A single i.c.v. injection in presymptomatic animals efficiently suppressed cytoplasmic TDP-43 aggregation, enhancing behavioral and locomotor abnormalities (Takeuchi et al., 2023).

3. Klotho

The pleiotropic nature of Klotho and its involvement in multiple processes including neuroprotection, remyelination, and modulation of neuroinflammation makes Klotho a very interesting molecule. Due to the inefficiency in treating ALS, we and others hypothesize that it may be necessary to act synergically by using a protein capable of intervening in as many pathways implicated in ALS as possible. Klotho fulfills these characteristics since it plays multiple roles in the CNS and has an excellent profile as a neurodegenerative and neuroprotective agent.

Klotho is an anti-aging hormone that was named after the Greek mythology goddess Clotho who visited each child after birth and controlled the length of the metaphoric thread of the human lifespan, deciding the fate of each child in life (Kuro-o, 2011). The α -Klotho (KL) gene was discovered by Kuro-O et al. (1997) after insertional mutagenesis events that disrupted the gene and led to a syndrome resembling accelerated aging. KL knockout mice display multiple aging phenotypes such as growth retardation, short lifespan (2-3 months), hypokinesia (diminished motor activity), gait abnormalities, atrophy of genital organs and thymus, arteriosclerosis, osteoporosis, and ectopic calcification, among others (Fig. 7).

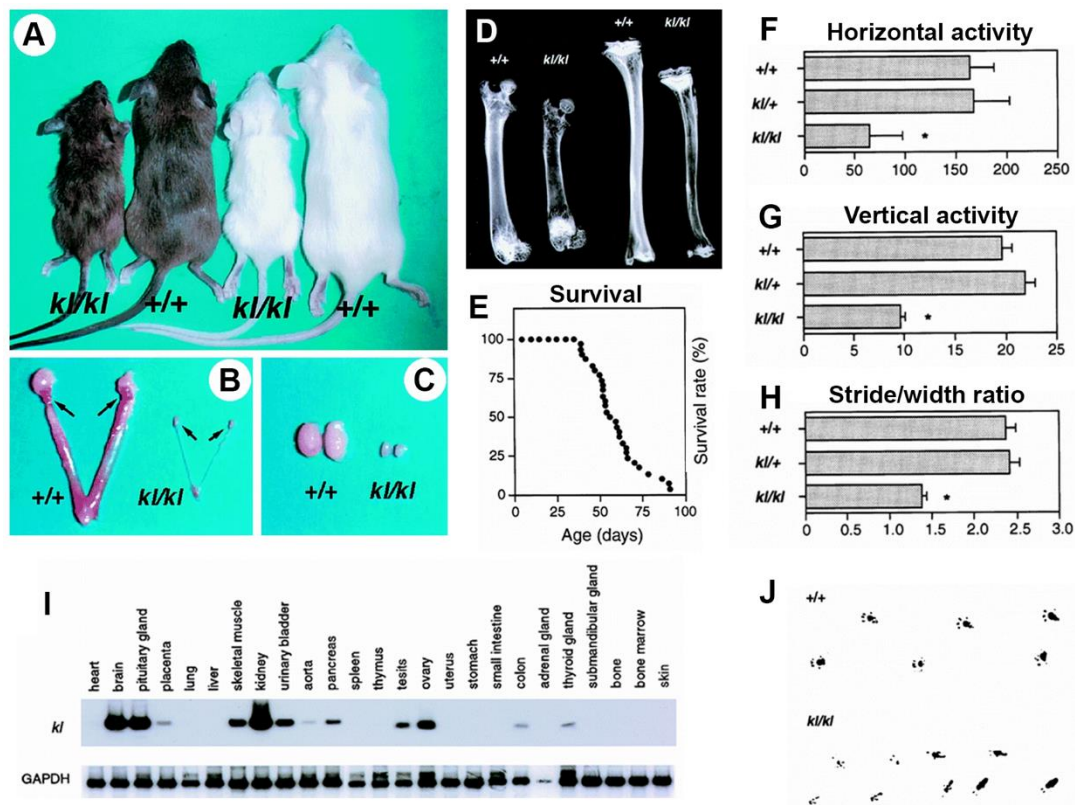


Figure 7. Aging phenotypes in Klotho knockout (kl/kl) mice. (A) kl/kl mice with c57BL/6j (agouti) and BALB/c (white) backgrounds with their +/+ littermates. Ovaries (arrows), uteri (B), and testes (C) are extremely atrophic in kl/kl mice. (D) Bone radiographs of femurs and tibiae indicate osteopenia. Decreased survival (E), horizontal (F), and vertical (G) activity and stride lengths in the hind-paw footprint test (H, J). (I) Klotho expression across tissues in wild-type mice. (Adapted from Kuro-O et al., 1997).

Since then, various functions have been attributed to this protein, displaying diverse anti-aging properties. Overexpression of KL can extend lifespan in wild-type mice by approximately 20-30% (Kurosu et al., 2005), whereas its silencing confers an extremely short survival (Masuda et al., 2005). Distinct transcripts and isoforms have been recognized, although a comprehensive understanding of the distinct properties or differences between these isoforms is still lacking.

3.1. Klotho gene and isoforms

The gene sequence of KL is remarkably conserved across humans, rats, and mice, demonstrating a high degree of sequence homology, reaching a 98% similarity between mice and humans. Additionally, the chromosomal location of the KL gene is also conserved, being found in humans (chromosome 13), rats (chromosome 12), and mice (chromosome 5) between the same genes (Y. Xu & Sun, 2015). Two other genes coding for proteins with shared homology to α -Klotho, β -Klotho, and γ -Klotho, have been identified (S. Ito et al., 2000, 2002). When the subfamily is not specified, the word "Klotho" typically refers to the α -Klotho subfamily, because it was discovered before the other members. KL is mainly expressed in the kidney and the brain, and to a lesser extent in the skeletal muscles, lung, urinary bladder, testes, and ovaries (Fig. 7I). β -Klotho is expressed in the liver, adipose tissue, and pancreas, whereas γ -Klotho is expressed in the kidney and skin. The KL promoter contains numerous CpG islands, regions susceptible to methylation that can suppress gene expression. This intrinsic methylation, occurring naturally, directs KL expression to specific organs. However, it is also responsible for the reduction in KL expression observed during the normal aging process (King et al., 2012).

KL is composed of five exons and four introns and has two different mRNA splice variants, the canonical codifying for a full-length transcript isoform (mKL, exons 1–5), of 3036 bp long in humans and 3042 bp in rats and mice (Fig. 8). mKL is a single-pass transmembrane protein (~130 kDa) composed of two extracellular domains, KL1 and KL2 (450 aa each). These domains can be shed by $\alpha/\beta/\gamma$ -secretases in the intracytoplasmic region to produce the processed soluble form (p-KL) or further cleaved between the extracellular domains to free pKL1 and pKL2 (Matsumura et al., 1998; Shiraki-Iida et al., 1998).

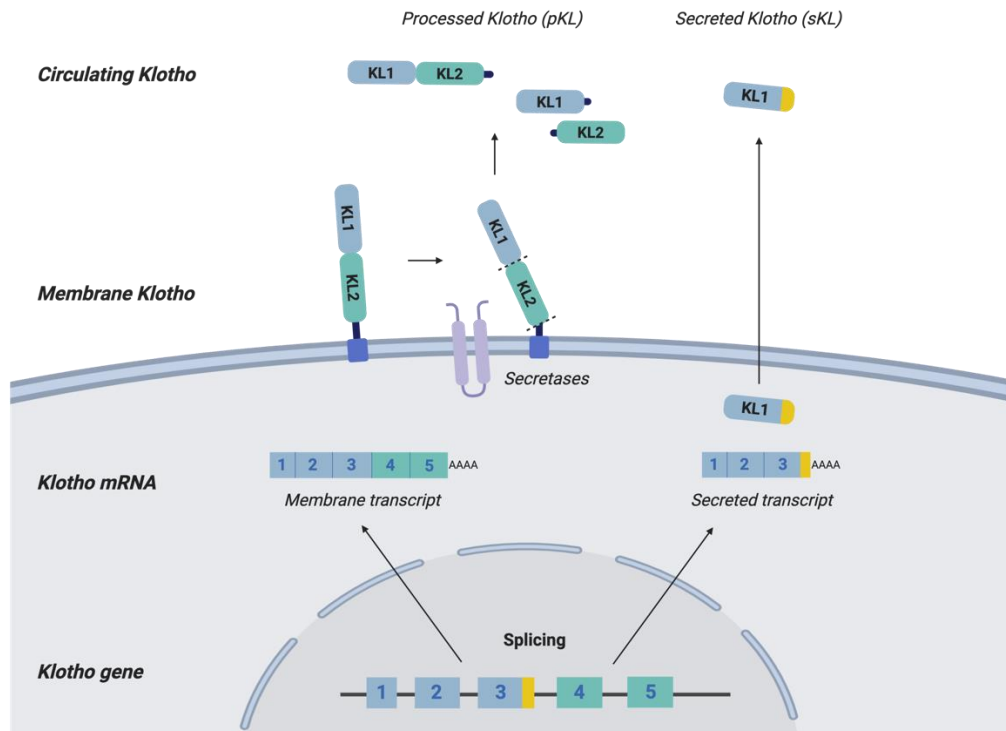


Figure 8. Processing of Klotho transcripts and isoforms. KL presents a full-length transcript that constitutes the transmembrane form (mKL), which can suffer proteolytic cleavage and generate processed KL forms. The alternative shorter transcript translates into a protein that includes a C-terminal secretion signal and is directly secreted to the extracellular space (Designed with Biorender, adapted from Prud'homme et al., 2022).

The second variant presents a STOP codon after the third exon and codifies for a secreted but shorter isoform of 550 aa (sKL, ~65 kDa), containing the KL1 domain (exons 1 to 3) plus an extra 15aa-tail found in the intronic region (Bian et al., 2015). This sKL transcript is 1650 and 1647 bp long in humans and mice, respectively, but has not been identified in rats. The KL1 and KL2 domains share similarities with the glycosidase protein family 1, which are enzymes capable of breaking down glycosidic bonds found in carbohydrate chains. KL has changes in essential amino acids within the predicted catalytic site of this protein family. Specifically, it lacks two crucial glutamic acids necessary for enzymatic activity.

Despite this, it appears to maintain a limited β -glycosidase activity (Tohyama et al., 2004). Furthermore, the amino acid sequence of KL contains multiple sites for N-glycosylation. Akin to glycosidase proteins, KL can self-associate and form dimers (Fig. 9B, C), creating oligomers representing the quaternary protein structure of KL (Imura et al., 2004).

Debate exists regarding the translation of this second transcript in humans as the lack of suitable antibodies has hampered studies of KL at the protein level. Some authors have not detected the secreted form in sera and CSF and proposed that this shorter transcript might not undergo transcription and could instead undergo nonsense-mediated decay (NMD) in humans (Imura et al., 2004; Mencke et al., 2017).

In mice, both variants have different expression levels as well as spatio-temporal expression profiles, suggesting different roles (Massó et al., 2015). The kidney shows the highest expression levels of KL, particularly in the distal tubules of the nephrons, being the main secretor of the hormone to the circulation. Specific knockout in this tissue can lead to similar phenotypes as in total KL deficient (*kl/kl*) mice (Olauson et al., 2012). Additionally, the parathyroid gland, choroid plexus, and sinoatrial node are identified as tissues with significant KL expression. The choroid plexus, referred to as “the kidney of the brain”, is lined by ependymal cells covering the brain ventricles' lumen and is responsible for producing the CSF.

Systemic administration of KL does not cross the BBB as measured by autoradiography and His-tagged protein studies (Hu et al., 2016; Leon et al., 2017). Thus, KL produced by these cells, which is believed to be primarily the secreted isoform, allows diffusion throughout the entire CNS (Lindberg et al., 2014). This is the basis for the proposed hormonal role of KL, processed and secreted KL are forms present in the bloodstream and CSF, exerting biological actions on distant organs and multiple systems.

Interestingly, the ratio of sKL mRNA to mKL mRNA is notably elevated in the brain compared to other organs, particularly in humans compared to mice (Massó et al., 2015; Matsumura et al., 1998). This indicates that soluble forms of KL could hold greater significance in the brain.

3.2. Klotho mechanisms of action

The KL protein is engaged in a wide array of processes, functioning both as a membrane-bound protein and as soluble variants (pKL, pKL1, pKL2, and sKL). The transmembrane form of KL plays a crucial role in mineral metabolism, acting as an obligate co-receptor for the fibroblast growth factor 23 (FGF23) hormone.

Additionally, soluble KL forms are integral in the regulation of mineral metabolism. They directly influence ion channels through their glycosidase activity and play a role in the hormonal regulation of parathyroid hormone (PTH) production, activation of vitamin D, and FGF23 hormone production (Dalton et al., 2017; Smith et al., 2012). However, the exact receptor mechanism for the soluble proteins remains elusive (Fig. 9). A suggested signaling pathway involves the interaction with lipid rafts, microdomains within the cytoplasmic membrane rich in interactions among various sphingolipids, cholesterol, and specific proteins. These lipid interactions modify membrane properties, facilitating specific signal transduction, cell adhesion, and molecular trafficking events. Sucrose gradient ultracentrifugation experiments revealed the simultaneous movement of pKL along with the lipid rafts fraction.

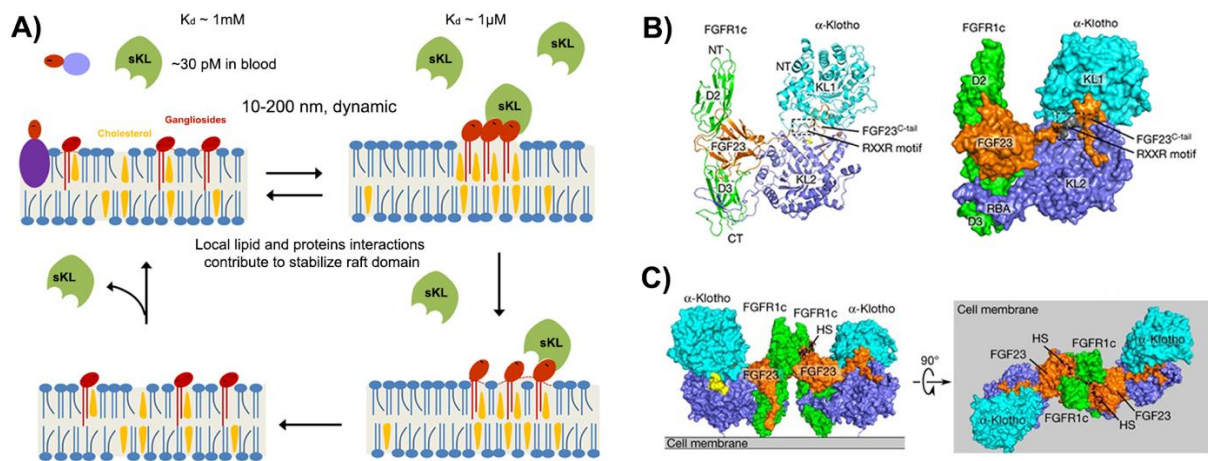


Figure 9. KL structure and their primary receptor mechanisms. (A) Model for sKL regulation of lipid rafts. Due to low concentrations and low affinity sKL does not bind to isolated gangliosides, but clustering in lipid rafts favors sKL binding (Adapted from Dalton et al., 2017). (B) Cartoon and surface representation of the ternary structure of mKL acting as a co-receptor of FGF23 to FGFR1. (C) Molecular surface of the quaternary dimers that are formed between mKL, FGF23, and FGFR1 (from Chen et al., 2018).

This interaction was also supported by findings from Förster resonance energy transfer (FRET) experiments and fluorescence microscopy, which demonstrated changes in membrane properties following pKL interaction within the lipid rafts (Dalton et al., 2017). Subsequent investigations have identified strong binding of the KL1 domain independently to lipid rafts, specifically to the $\alpha 2,3$ -sialyllactoses region of the gangliosides GM1 and GM3 (Wright et al., 2017). This interaction of KL1, and therefore of all KL soluble forms, alters the trafficking of molecules and signals through glycan-binding proteins. Moreover, Dalton et al. (2017) also demonstrated that KL can activate the PI3/Akt pathway by modulating lipid rafts.

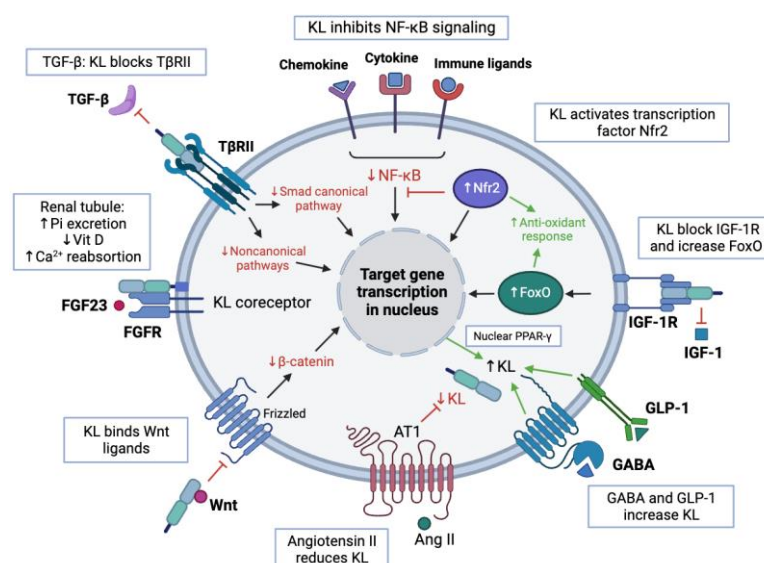


Figure 10. Multiple functions of Klotho in signaling pathways (Adapted from Prud'homme et al., 2022).

3.1.1. Klotho in mineral metabolism

Serving as a co-receptor for the FGF receptor, KL actively participates in FGF23 signaling and the regulation of phosphate (Pi) concentration. In response to increased Pi in circulation, FGF23 is secreted by osteocytes to reduce Pi levels by increasing renal excretion and by decreasing the amount of active vitamin D in the bloodstream. Vitamin D increases renal and intestinal Ca^{2+} and Pi absorption but also upregulates KL transcripts and FGF23 production (Forster et al., 2011), which in turn inhibit further vitamin D synthesis, closing the Pi concentration regulatory loop (Tsujikawa et al., 2003). KL forms a stable interaction with the fibroblast growth factor receptor (FGFR) through the KL2 domain, mediating signaling via the ERK1/1 MAPK pathway.

Notably, as KL is linked to FGF23 production, administering exogenous pKL significantly increases FGF23 levels in serum, impacting mineral metabolism (Smith et al., 2012). In a previous work, we have demonstrated that excessive systemic overexpression of pKL, but importantly not of sKL, has detrimental effects on mineral metabolism and bone microstructure (Roig-Soriano et al., 2023). This raises a safety concern for treatments aiming to elevate full-length KL protein concentration exogenously.

KL also plays a significant role in regulating Ca^{2+} levels by modulating various ion channels and regulating the production of the PTH in the parathyroid glands (Pavlatou et al., 2016). Parathyroid glands express high levels of KL, where it functions as a co-receptor for FGF23 in response to elevated Ca^{2+} levels. The parathyroid gland monitors the extracellular calcium concentration via, secreting PTH hormone to maintain appropriate levels. In KL-expressing organs, KL directly influences specific ion transporter proteins, accumulating them in the ER and Golgi apparatus, and rapidly augmenting their abundance and activity in the cellular membrane when necessary. Activation of this channel creates an electrochemical gradient in the membrane, triggering vital processes such as enhanced ATPase activity and the recruitment of ion channels (Nabeshima & Imura, 2008).

3.1.2. Inhibition of the insulin/IGF-1 pathway

One of the primary mechanisms through which KL exerts its anti-aging effects and promotes life expectancy is through the inhibition of the insulin/IGF-1 pathway. Within pancreatic cells, it is involved in the secretion of insulin hormone (Lin & Sun, 2012, 2015). Conversely, insulin also modulates KL function, as it induces shedding and dependent solubilization of KL. Additionally, KL acts as a negative regulator of Insulin Receptor Substrates (IRS) within the downstream metabolic cascade of insulin (IR) and IGF-1 receptors. This inhibitory effect of KL has been demonstrated through studies on KL knockout models, revealing reduced insulin production and enhanced

insulin sensitivity (Utsugi et al., 2000). These positive impacts are in part due to the suppression of the Forkhead Box (FOXOs) transcription factor phosphorylation, a process that occurs post-insulin/IGF-1 activation and that induces its translocation from the nucleus. FOXOs govern the expression of key factors implicated in apoptosis, cell cycle, resistance to oxidative stress and metabolism, and KL aids in maintaining its transcriptional activity (Akintola & van Heemst, 2015).

3.1.3. Wnt/ β -Catenin signaling inhibition

The KL1 domain can interact with several Wnt ligands (Wnt1, 3, 4, and 7a), pivotal proteins in the downstream signaling of the Wnt/ β -Catenin signaling pathway, effectively impeding its signal transduction. The activation of the Wnt pathway plays a crucial role in cell proliferation and division of stem cells; however, prolonged, or excessive activation of this pathway is linked to premature stem cell depletion. Additionally, sustained activation of the Wnt pathway has been associated with tissue fibrosis (L. Zhou et al., 2013). KL-mediated inhibition of the Wnt signaling, potentially explaining the benefits of KL in mitigating pathological fibrosis, especially in conditions like muscle dystrophy or injury, chronic kidney disease, and aging (Wehling-Henricks et al., 2016; Welc et al., 2020; Zhou et al., 2013).

3.1.4. Antioxidative effects of Klotho

Resistance to oxidative stress is a mechanism that may explain KL-mediated suppression of aging. This was first described in KL-overexpressing mice, measured as low urinal levels of the oxidative damage marker 8OH²dG. Soluble KL inhibition of insulin/IGF-1/PI3K signaling results in the activation of FOXO transcription factors and the upregulation of antioxidant enzymes, such as catalase, the manganese superoxide dismutase (MnSOD), Prx-2/3 and other members of the thioredoxin/peroxiredoxin (Trx/Prx) system, all important for removing ROS (Yamamoto et al., 2005; Zeldich et al., 2014). Soluble KL also attenuates superoxide production and reduces H₂O₂-induced cell senescence and apoptosis by inhibiting the caspase 3/9 and p53/p21 pathways (de Oliveira, 2006; Ikushima et al., 2006). In the brain, KL has been shown to protect dopaminergic neurons from oxidant-induced degeneration by modulating the ROS-sensitive apoptosis signal-regulating kinase 1 (ASK1) and the p38 MAPK pathway (Brobey et al., 2015).

Another mechanism by which KL mitigates ROS production entails maintaining the structural integrity of mitochondria and their optimal bioenergetic capacity. KL absence leads to cardiolipin content depletion, compromised cristae structure, and vacuolation of mitochondria. Cardiolipin is an anionic phospholipid predominantly located in the inner mitochondrial membrane where it is

synthesized. It is essential for the assembly and optimal functioning of mitochondrial respiratory supercomplexes. This loss of structure can lead to cardiolipin reorganization and oxidation, transforming CytC into a peroxidase that generates oxidants (Sahu et al., 2018). KL also activates peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC1 α), the master regulator of mitochondrial biogenesis, via the LKB1-AMPK-PGC-1 α pathway (Lee et al., 2021).

3.1.5. Klotho's anti-inflammatory profile

KL has been associated with immunomodulatory processes through various pathways. In primary macrophage cultures, KL inhibited the activation of the NLRP3 inflammasome by enhancing FGF 23 signaling (Zhu et al., 2018). Additionally, KL has shown the ability to inhibit NF- κ B activation (Maekawa et al., 2009), suppressing the production of inflammatory cytokines in response to TNF- α stimulation (Maekawa et al., 2009), and increasing levels of anti-inflammatory cytokines like IL-10 (Mytych et al., 2018). In a recent study, the overexpression of both sKL and mKL in a mouse model of accelerated aging corroborated these findings. Glial cell counts in the brain were restored to normal levels. The pro-inflammatory cytokines activated by TNF- α were downregulated, with a tendency for anti-inflammatory cytokines to increase (Roig - Soriano et al., 2022). Finally, in a large cohort of human sera, soluble KL exhibited a negative correlation with uric acid, c-reactive protein, and white blood cell count, established inflammatory biomarkers. Reduced soluble KL levels imply a general inflammatory state, making KL a potential biomarker inversely linked with inflammatory conditions (Wu & Chen, 2022).

3.1.6. Klotho in excitotoxicity

Although there is limited available data, KL has demonstrated its capacity to shield cells against glutamate-induced cytotoxicity. When cultured hippocampal neurons are exposed to high concentrations of glutamate, KL fosters neuronal survival by activating the PI3K/Akt pathway and regulating the Trx/Prx redox system (Zeldich et al., 2014). Additionally, β -Klotho, which shares a ~42% homology with α -Klotho, increases the expression of the excitatory amino acid transporters. Consequently, it enhances their presence in the cell membrane and the maximum transport rate, thereby contributing to glutamate clearance and the regulation of neuronal excitation (Almilaji et al., 2013; Warsi et al., 2015).

3.1.7. Klotho in ER stress and autophagy

Some *in vitro* studies show that klotho has also the ability to ameliorate chemically induced ER stress. KL overexpression resulted in a reduction of ER stress and UPR markers. These markers include phospho-inositol requiring enzyme 1 (IRE1), X-box binding protein 1 (XBP-1s), binding immunoglobulin protein (BiP), CHOP, phospho-c-Jun N-terminal kinase (JNK), and phospho-p38, all of which were initially elevated (S. Banerjee et al., 2013; Cui et al., 2018). KL also suppressed caspase and poly (ADP-ribose) polymerase (PARP) cleavage, leading to enhanced cell viability. On the contrary, siRNA-mediated knockdown of KL worsened ER stress signaling.

In human fibroblasts, Mytych and collaborators (2019) studied KL silencing combined with exposure to a non-toxic dose of lipopolysaccharide (LPS). Suppressing KL expression resulted in a notable rise in caspase 3 activity and a reduction in the Bcl-2 protein, suggesting activation of the apoptotic pathway and cytotoxic autophagy. Calnexin, which is associated with apoptosis induced by ER stress, also showed a significant increase in its expression after KL depletion and LPS treatment. Lastly, cells treated with LPS and without KL knockdown exhibited a marked increase in the activation of protective autophagy. Overall, these studies indicate that KL is involved in both ER-mediated stress responses and autophagy.

3.1.8. The role of Klotho in myelination

A role for KL in myelination and oligodendrocyte biology was first reported after the discovery of reduced myelin expression in the white matter of aged rhesus monkeys and the discovery of myelination abnormalities in KL-knockout mice (Chen et al., 2013; Duce et al., 2008). KL enhances oligodendrocyte maturation and myelination by activating Akt and ERK1/2 and upregulating myelin proteins (Chen et al., 2014). On the contrary, KL-deficient mice show decreased myelin-related proteins, a lower percentage of myelinated fibers, and impaired myelin at the nodes of Ranvier (Chen et al., 2013). In addition, KL can stimulate spontaneous remyelination both in cuprizone-induced demyelination and in the experimental autoimmune encephalomyelitis (EAE) model (Zeldich et al., 2015).

3.1.9. Modulation of senescence

Cellular senescence is a condition where cells halt their division and growth while maintaining metabolic activity. This natural and irreversible process is triggered in cells after a specific number of divisions or due to diverse stressors. However, an overabundance of senescent cells can foster

persistent inflammation and impair tissue function. *In vitro* experiments inducing downregulation of KL resulted in premature onset of senescent phenotype, which was marked by higher senescence-associated β -galactosidase (SA β -Gal) activity and an increase in the p53/p21 senescence signaling pathway (de Oliveira, 2006).

Conversely, elevating KL levels led to a reduction in H₂O₂-induced senescence and apoptosis, which was also linked to a decreased expression of p53/p21 (Ikushima et al., 2006). The KL ectodomain can inhibit TGF β -R2, a receptor crucial for TGF- β 1 signaling. TGF- β 1 is known for its dual role in inflammation, exhibiting both pro-inflammatory and anti-inflammatory functions and playing a significant role in fibrotic processes. During prolonged inflammatory conditions, TGF- β 1 stimulates chemotaxis of fibroblasts and encourages the secretion of extracellular matrix proteins while inhibiting proteases, ultimately contributing to chronic fibrosis (Doi et al., 2011).

Human iPSC-derived cortical neurons, in 2D cultures or in 3D cortical organoids, develop typical hallmarks of senescent cells when maintained *in vitro* for prolonged periods. Moderate upregulation or repression of endogenous KL in cortical organoids inhibits and accelerates senescence, respectively. More importantly, KL inhibits neuronal senescence and alters the expression of senescence-associated genes including, extracellular matrix factors, and proteoglycans.

In a senesce mouse model, AAV-mediated overexpression of sKL and mKL diminished β -galactosidase activity and its accumulation in the cortex, with lower p16 and p21 expression levels (Roig - Soriano et al., 2022).

3.1.10. Klotho protection from cognitive decline

KL plays a crucial role in modulating synaptic activity, particularly in ensuring the proper functioning of the cholinergic and dopaminergic neuronal systems. This understanding stems from research on KL-knockout mice, which exhibited abnormal expression of proteins crucial for acetylcholine production and reception (Park et al., 2013). Furthermore, these mice demonstrated deficiencies in the dopaminergic neuronal system, experiencing premature death of TH-positive neurons, responsible for dopamine secretion. Vitamin D-dependent toxicity, exacerbated in the absence of KL protein, partly contributed to this phenotype.

In behavioral tests involving long-term memory, *kl/kl* mice exhibited reduced performance but displayed no alterations in anxiety, exploratory behaviors, or short-term memory levels (Nagai et al., 2003). Additionally, these mice showcased fewer synapses and increased apoptosis in the hippocampus. Conversely, experiments involving mice overexpressing KL demonstrated improved

cognition and enhanced performance in various memory tests. This improvement was linked to heightened expression of the GluN2B subunit of the NMDA receptor, a critical component during the long-term potentiation process of memory formation (Dubal et al., 2014). In a senescence-accelerated mouse model, sKL and mKL overexpression improved cognitive performance in a battery of tests, without changes in age-related behaviors (Roig-Soriano, 2022).

Systemic KL also augments neural resilience to Alzheimer's and Parkinson's disease-related toxicities (Baluchnejadmojarad et al., 2017; Dubal et al., 2015; Leon et al., 2017; Zeng et al., 2019), which is supported by other studies demonstrating that individuals with elevated KL have a decreased risk of dementia (Ali et al., 2022; Belloy et al., 2021; Driscoll et al., 2021; Grøntvedt et al., 2022). Building upon this, Castner and collaborators (2023) have recently sustained this concept in aged NHPs. A single intravenous administration of sKL protein showed increased synaptic plasticity and cognition. Interestingly, these outcomes outlasted the expected half-life of the hormone, indicating organizational, persisting, and beneficial effects on the synapse and brain. Given that systemically injected KL does not penetrate the brain, these advantageous effects must be orchestrated by peripheral messengers that transmit its signals to the brain.

3.1.11. Muscle and myofiber regeneration depend on Klotho

Muscle and myofiber regeneration seem to highly depend on KL expression. Recent studies demonstrate that KL decline drives dysfunctional MPC and myogenesis. *Kl/kf* mice have extensive fibrosis, scarring, and calcification after acute muscle injury, while KL-overexpressing mice show increased muscle growth and improved regeneration (Ahrens et al., 2018; Welc et al., 2020). In the *mdx* mouse model of Duchenne muscular dystrophy, KL undergoes muscle-restricted epigenetic silencing, and its overexpression restores longevity, reduces muscle wasting, and improves mouse function (Wehling-Henricks et al., 2016). Following injury, wild-type young skeletal muscles display a local increase of KL with transient demethylation of its promoter, a phenomenon lost with aging. Genetic ablation of KL in young MPCs drove mtDNA damage and decreased cellular bioenergetics, like in aged animals. Conversely, supplementing with KL reinstated mtDNA integrity and bioenergetics in aged MPCs to youthful levels, both *in vitro* and by boosting the functional regeneration of aged muscles *in vivo*. Youthful levels of KL appear to be critical for the maintenance of mitochondrial ultrastructure, limiting mtDNA damage and mitochondrial ROS production, which is necessary for MPC activation and contribution to functional myofiber regeneration (Sahu et al., 2018b).

Interestingly, the same authors conducted heterochronic blood exchange (HBE) experiments between young and aged mice and showed that the beneficial effect of young blood on aged

muscle regeneration was diminished when serum was depleted of extracellular vesicles (EVs). Machine learning classifiers demonstrated that aging alters the nucleic acid signature of circulating EVs. Transcriptomic profiling identified KL among the candidates that are abundant in young but not in old EVs. Significantly, the administration of EVs contributed to functional skeletal muscle regeneration in a KL mRNA-dependent manner (Sahu et al., 2021).

3.2. Klotho and ALS: previous evidence

Certain studies point towards a beneficial effect of KL in ALS, such as in other neurodegenerative disorders. KL knockout mice show altered gait and, despite having similar numbers of MNs in the ventral spinal cord, they have less large and Nissl-positive neurons. The frequency distribution of the sectional area of the neuronal soma and the nucleoli diameter in the ventral gray matter confirmed a shift towards a smaller size. Astrogliosis and neurofilament phosphorylation are also evident in the ventral horn of KL-lacking mice (Abraham et al., 2016; Anamizu et al., 2005a; Kurosu et al., 2005). These changes in anterior horn cells (AHCs) resemble MN degeneration seen in ALS, with the apparent chromatolysis described in patients (Martin, 1999).

ALS-like NFL accumulation and impaired axonal transport of cytoskeletal proteins were also observed in large, myelinated axons of the sciatic nerve (Uchida et al., 2001). KL-deficient mice had a significant reduction of cytoplasmic RNA in AHCs, with fragmented rough ER and fewer attached and free ribosomes. These findings imply that KL deficiency leads to the shrinkage and impaired function of MNs, due to a reduction in ribosomal RNA (rRNA) gene transcription activity (Anamizu et al., 2005b).

To assess the direct therapeutic potential of KL in ALS, researchers conducted a study where mice overexpressing KL were bred with SOD1^{G93A} mice. The overexpression of KL resulted in a delay in disease onset, a deceleration of disease progression, and an extension of survival, with a significant effect only in the female group. Elevated KL levels also preserved grip strength in mSOD1 mice. The gene expression profile of KL x SOD1^{G93A} mice in the motor cortex and lumbar spinal cord showed increased levels of the antioxidative enzymes Prx-2 and Prx-3 and their upstream regulator Nrf2, as well as for myelin-associated glycoprotein (MAG) and myelin basic protein (MBP). The expression of the proinflammatory proteins TNF α , TNFAIP2, IL-1 α , IL-1 β , and IL-12a was downregulated. KL overexpression in bigenic mice also reduced the number of Iba1-positive cells and fostered neuronal rescue, being more dramatic in the lumbar areas of the spinal cord (Zeldich et al., 2019).

A wealth of evidence strongly backs the notion that high KL levels lower susceptibility to neurodegenerative processes. Given its multifaceted nature, treatment with KL holds the potential to ameliorate various of the underlying pathological mechanisms in ALS (Fig. 11).

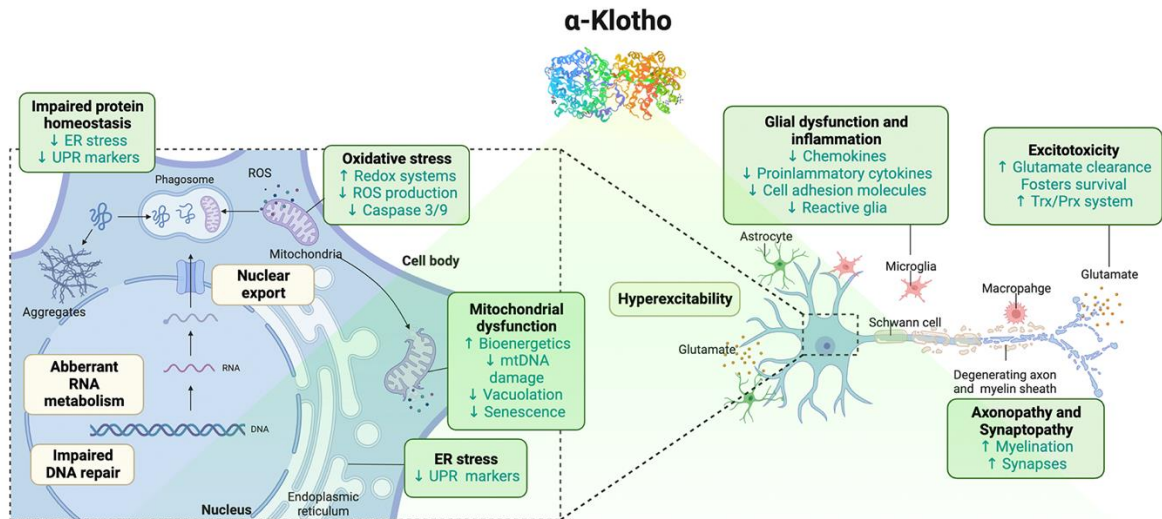


Figure 11. Possible roles of Klotho in the pathophysiological mechanisms of ALS.

HYPOTHESIS AND OBJECTIVES

In this thesis, we hypothesized that α -Klotho participates in multiple events related to the etiopathogenesis of ALS, and is responsible for MN degeneration and muscle denervation. The general aim of the thesis is to demonstrate the therapeutic effects of secreted α -Klotho in ALS and study its different roles in the skeletal muscles and in the spinal cord. To that end, the following specific objectives are proposed:

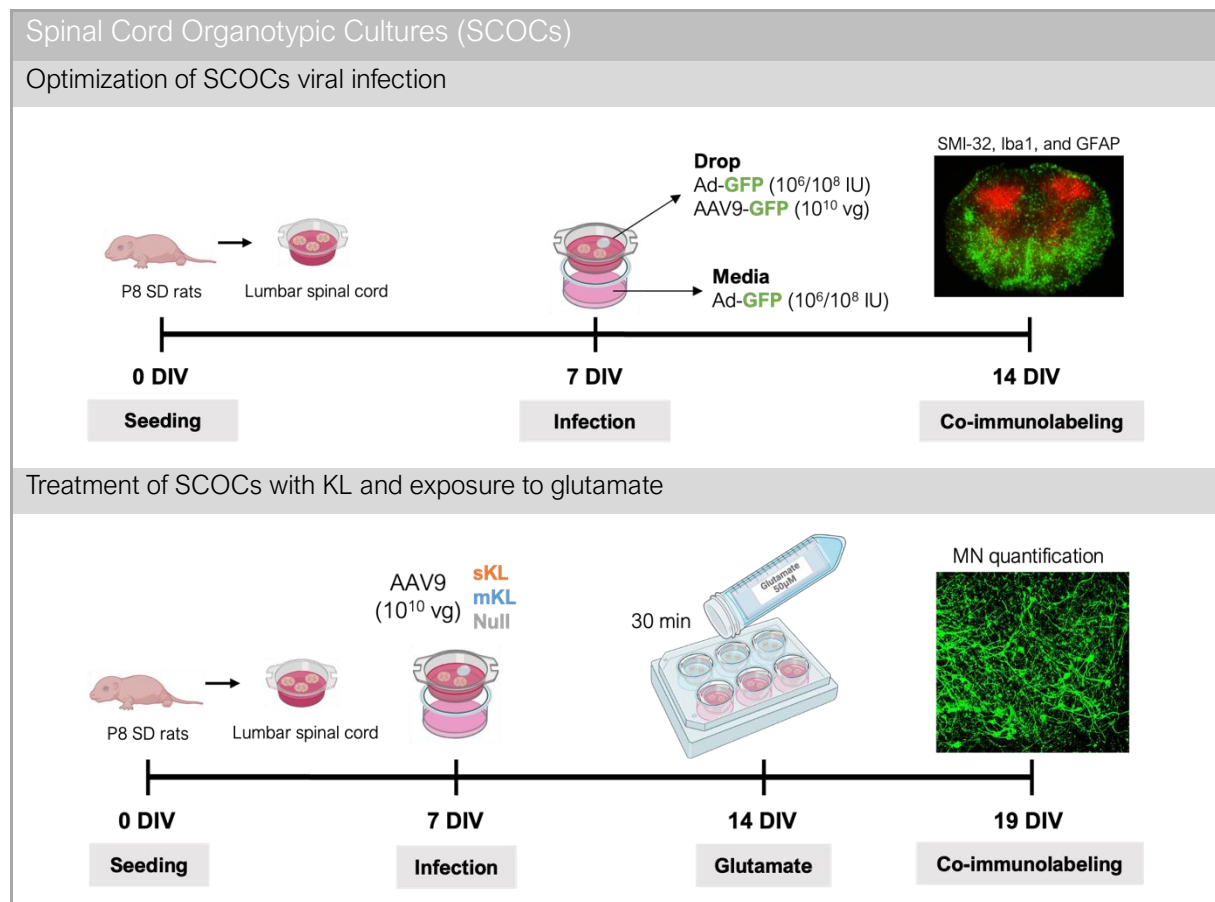
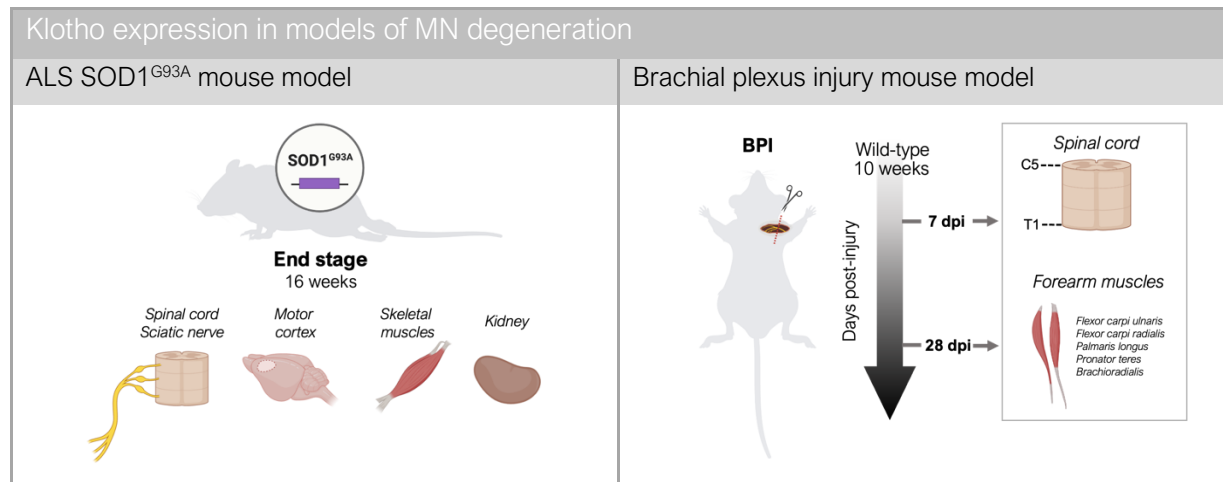
1. Study the expression of the different isoforms of α -Klotho in muscles, spinal cord, motor cortex, and kidney of SOD1^{G93A} mice and its relation to MN degeneration and muscle denervation.
2. Characterize the effect of α -Klotho on motoneuron survival in an *in vitro* model of spinal cord organotypic cultures.
3. Overexpress α -Klotho in the spinal cord of SOD1^{G93A} mice by gene therapy to protect motoneurons.
4. Overexpress α -Klotho in all the skeletal muscles of SOD1^{G93A} mice by gene therapy to preserve the innervation of neuromuscular junctions and avoid the dying-back of motoneurons.
5. Identify genes, proteins, and pathways that are key in the neuroprotective and myoregenerative properties of Klotho in ALS.

GRAPHICAL STUDY DESIGN

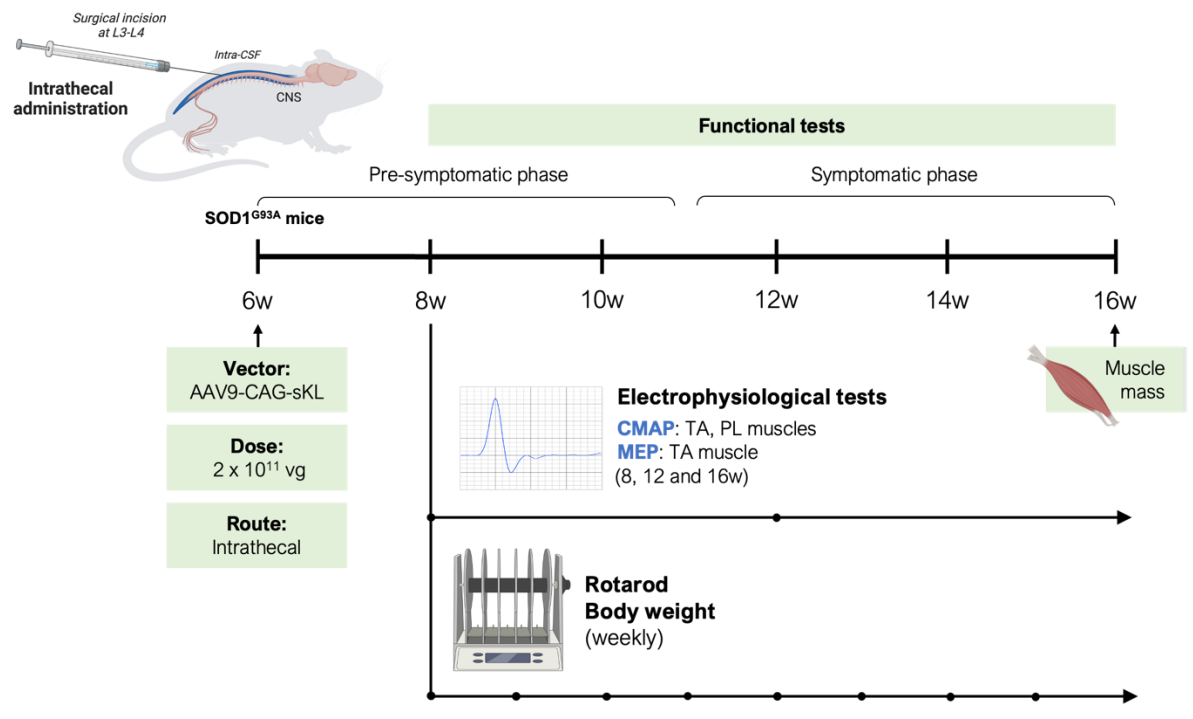
Chapter I

Validation of Klotho in models of motoneuron degeneration. Therapeutic overexpression of Klotho in the spinal cord of SOD1^{G93A} mice.

The opening chapter of the thesis is focused on characterizing the effect of MN degeneration and muscle denervation on KL levels. The effect of KL on MN preservation was directly evaluated *in vitro* and *in vivo* on experimental models of MN degeneration.



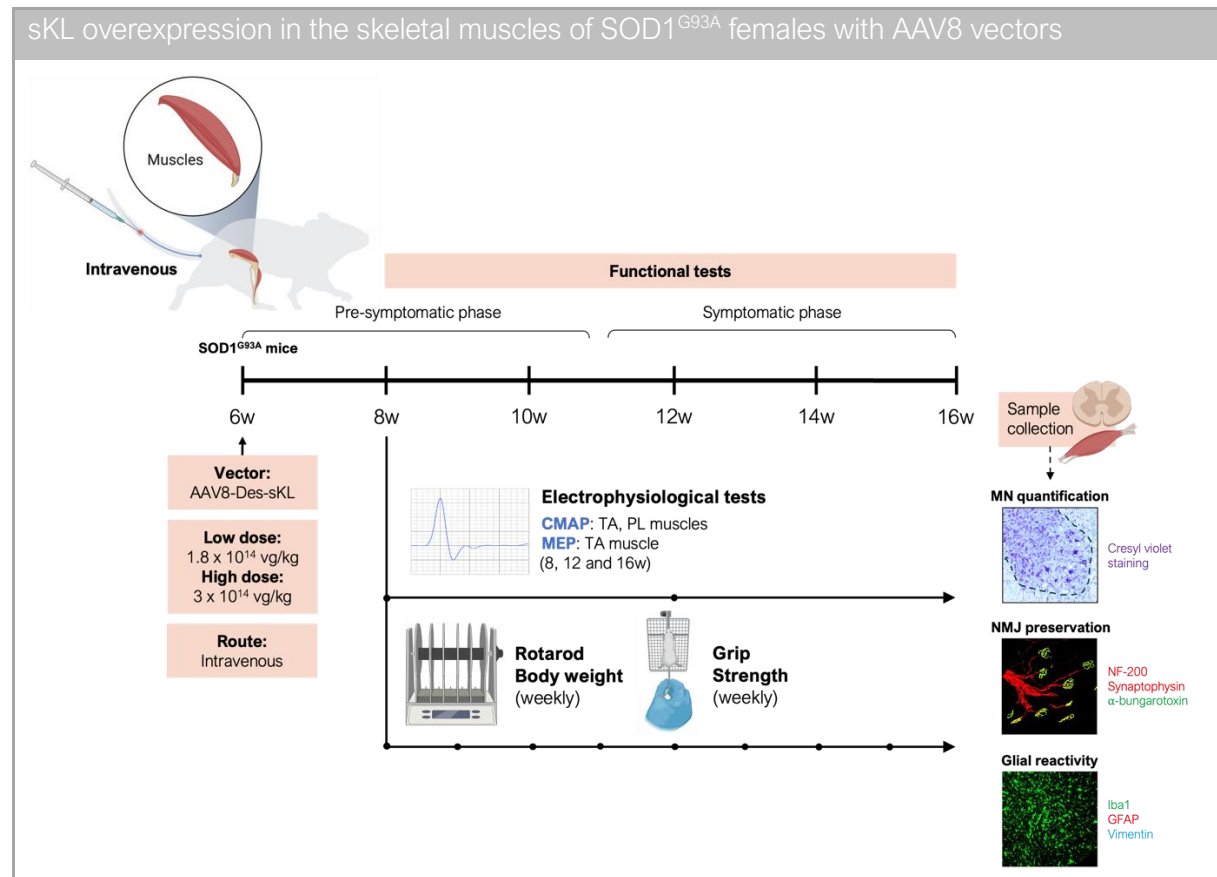
Secreted Klotho overexpression in the spinal cord of SOD1^{G93A} mice



Chapter II

Targeting skeletal muscle-specific overexpression of Klotho with systemic AAV8 vectors.

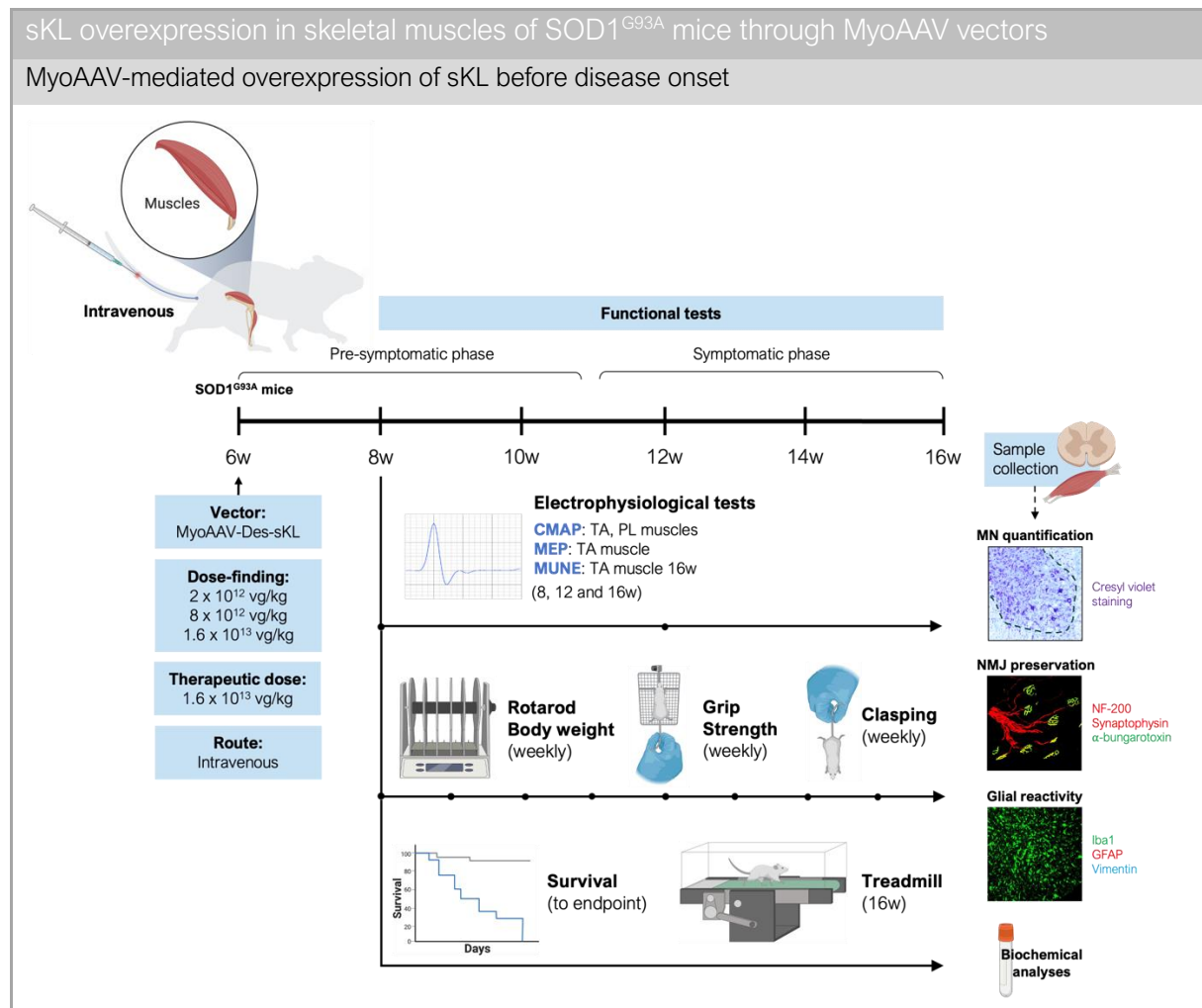
In the second chapter, we constrained KL overexpression to the muscles of SOD1^{G93A} mice using intravenously delivered AAV8 vectors and the desmin promoter. We investigated the secretion of sKL from muscles, assessing its potential to uphold neuromuscular and motor function, safeguard MNs, and preserve NMJs.



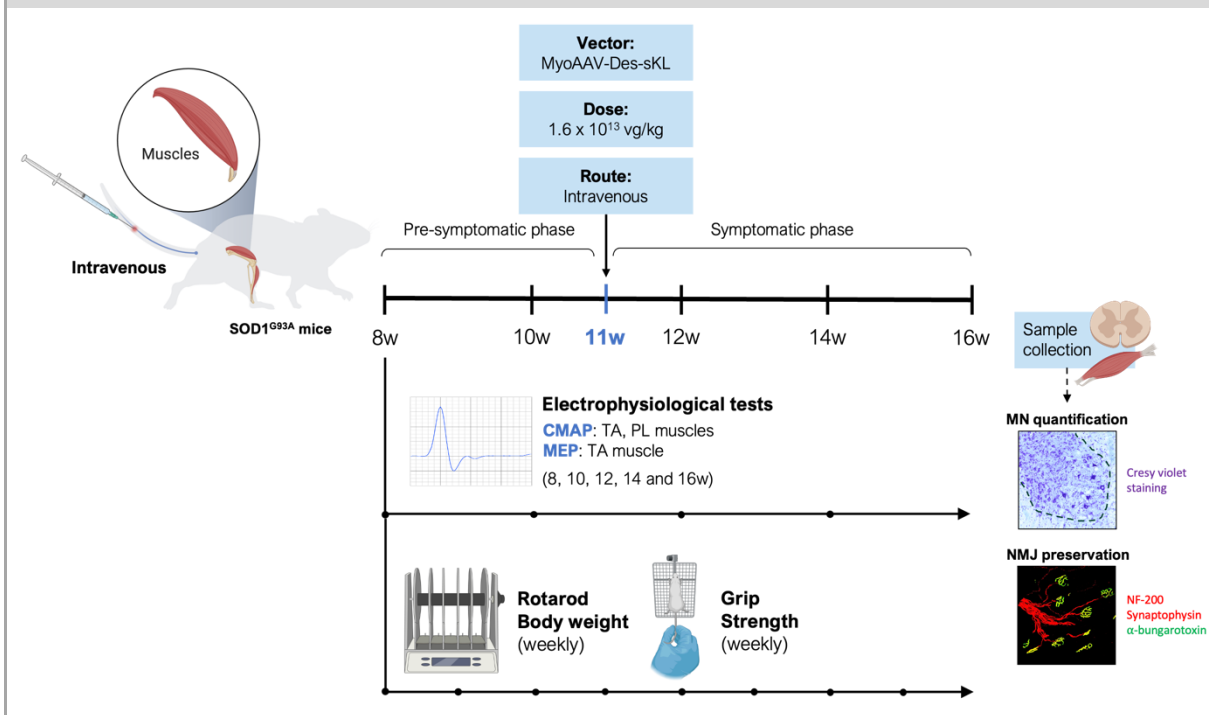
Chapter III

Enhanced Klotho delivery to muscles with the novel myotropic MyoAAV vector.

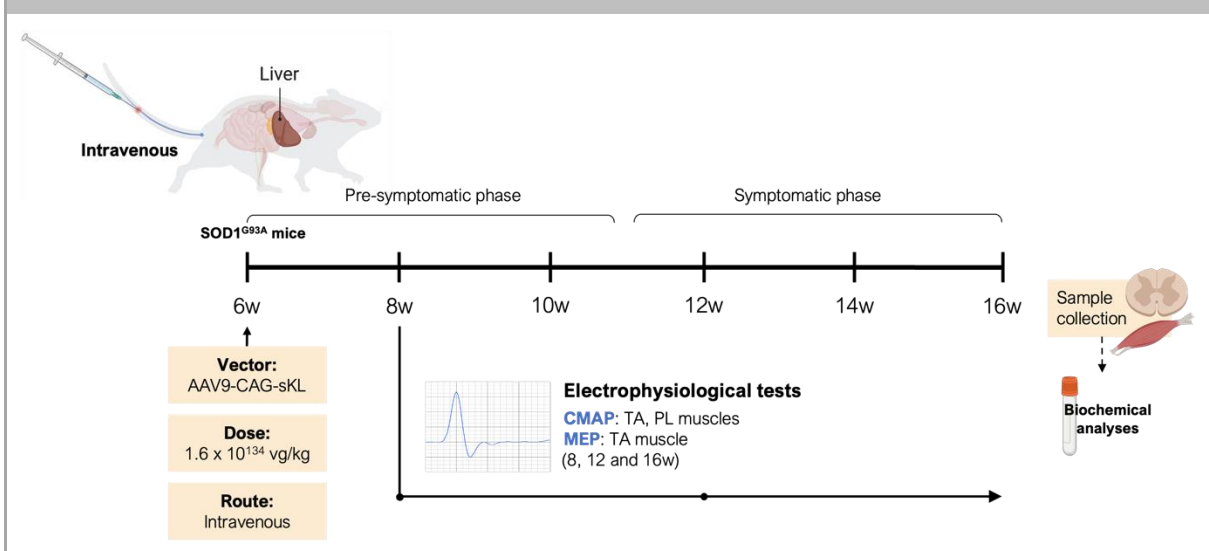
In the third chapter, our attention shifts to evaluating the capacity of a myotropic vector to achieve with a lower dose the therapeutic effects of sKL secretion from muscles in the context of ALS. We also examine the ability of sKL to decelerate disease progression during a symptomatic stage. Furthermore, we test the potential of widespread KL overexpression to preserve neuromuscular function, employing intravenous AAV9 vectors.



MyoAAV-mediated muscle overexpression of sKL at a symptomatic stage



Systemic overexpression of secreted Klotho in female SOD1^{G93A} mice

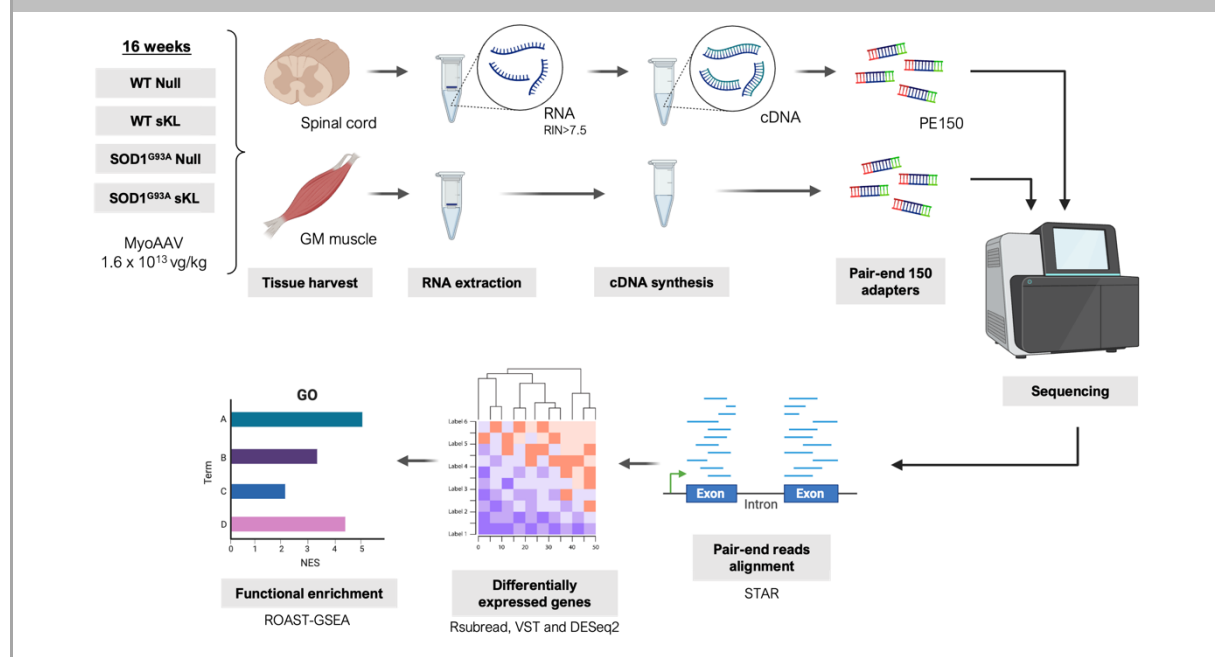


Chapter IV

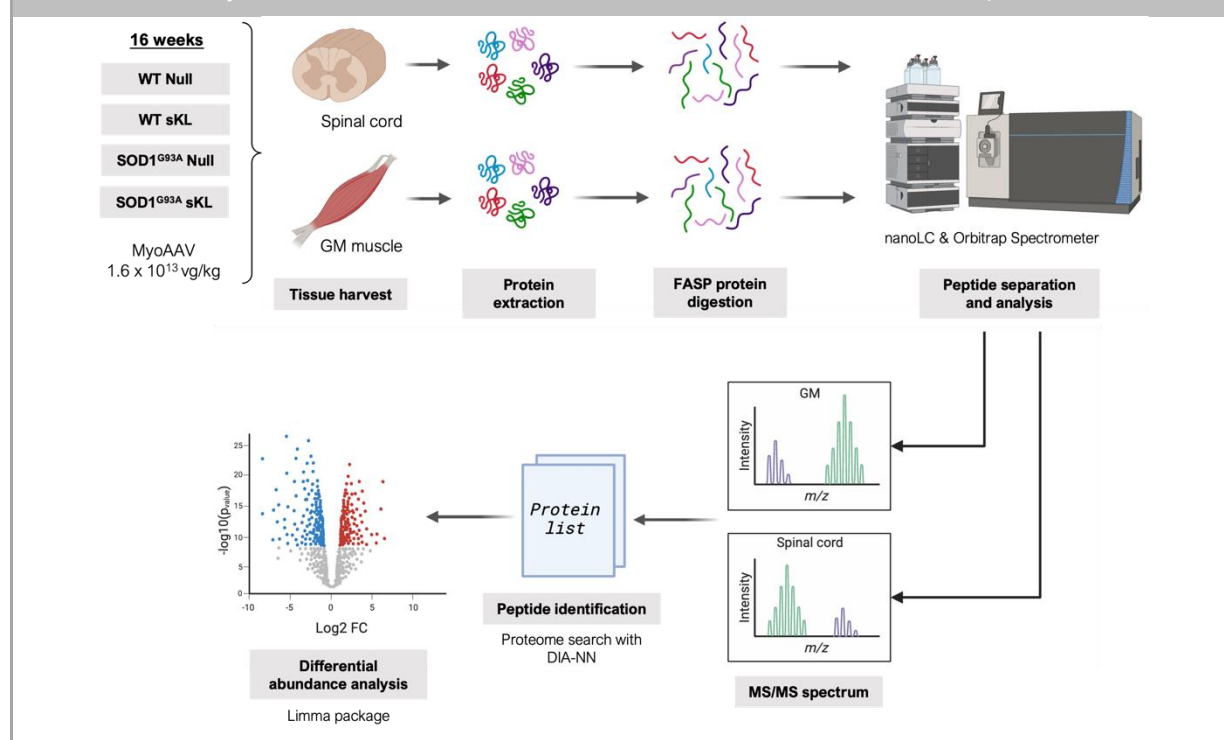
Transcriptomic and proteomic studies unravel Klotho mechanisms of protection in ALS.

In the final chapter, we delve into transcriptomic and proteomic studies to analyze the gene expression patterns in the spinal cord and gastrocnemius (GM) muscles of wild-type and SOD1^{G93A} animals overexpressing sKL in the muscles.

RNAseq workflow for sKL- and mock-treated WT and SOD1^{G93A} female samples.



Proteomics analysis of sKL- and mock-treated WT and SOD1^{G93A} female samples.



RESULTS

Chapter I:

Validation of Klotho in models of motoneuron degeneration. Therapeutic overexpression of Klotho in the spinal cord of SOD1^{G93A} mice

1.1. Klotho decreases in models of MN degeneration and muscle atrophy

To determine the endogenous expression of KL isoforms in ALS we performed quantitative real-time PCR on several tissues from SOD1^{G93A} mice at the advanced disease stage of 16 weeks. Previous studies from our group have demonstrated that KL isoforms show different spatiotemporal profiles of expression in aging and other neurodegenerative disorders (Massó et al., 2015). In SOD1^{G93A} mice, we observed a significant downregulation of secreted KL in the gastrocnemius and soleus muscles (0.28 ± 0.15 and 0.44 ± 0.08 , respectively) compared to wild-type mice of the same age (Fig. 12A). Interestingly, the motor cortex of the brain also exhibited a notable reduction in expression in these mice (0.39 ± 0.12 -fold-change). For transmembrane KL, we found that the mRNA levels of this isoform were strongly reduced in the three nervous tissues analyzed: motor cortex (0.54 ± 0.08), spinal cord (0.35 ± 0.06), and sciatic nerve (0.37 ± 0.04).

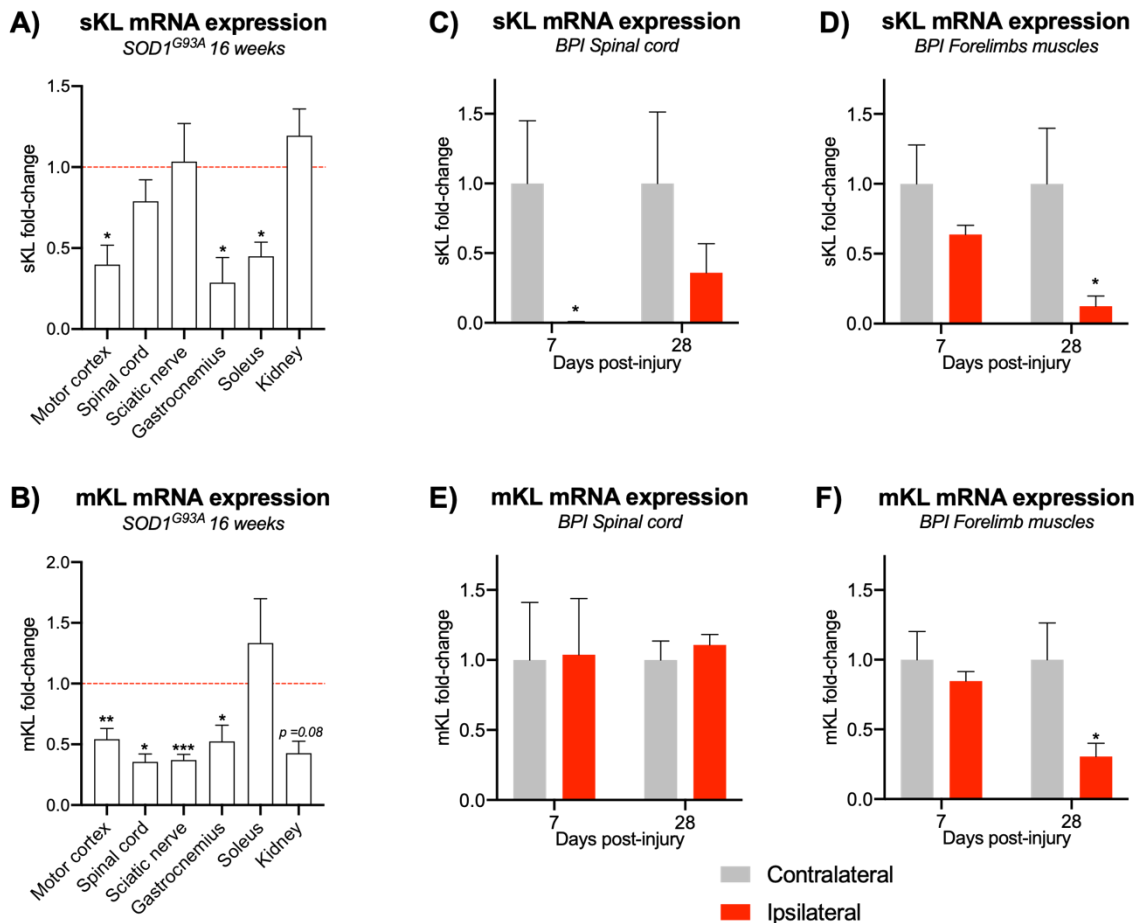


Figure 12. Klotho isoform expression in motoneuron degeneration models. Secreted (A) and transmembrane (B) Klotho expression in different tissues of the SOD1^{G93A} mouse model at a late phase of the disease. Dashed red lines represent normalized WT age-match levels for each tissue. sKL expression levels in the spinal cord (C) and forelimbs muscles (D) of the brachial plexus injury (BPI) model at 7- and 28-days post-lesion. mKL expression in the BPI model for the same tissues and days post-injury (E, F). One-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; *vs WT or contralateral side. Data is shown as mean $\pm$ SEM ($n = 6-8$ for expression in SOD1^{G93A} and 4-5 in BPI studies).

In the muscles, mKL expression was decreased in the gastrocnemius (0.52 ± 0.13) but remained unchanged in the soleus (Fig. 12B). Notably, in the kidney, which serves as the primary source of circulating KL, there was a tendency for mKL to be downregulated (0.42 ± 0.09).

To elucidate if the downregulation of KL is directly linked to the SOD1^{G93A} mutation and the underlying mechanisms of ALS or if it is a result of muscle atrophy and motoneuron degeneration, we investigated the model of brachial plexus injury (BPI) at 7- and 28-days post-injury (dpi). In this model, the connection between the C5-T1 spinal cord segments and the muscles of the forelimbs is disrupted. In the corresponding ipsilateral spinal cord segment, sKL levels were markedly decreased at 7-days compared to the contralateral side (0.006 ± 0.003), and although not significantly, sKL levels remained low at 28 dpi (0.35 ± 0.2 , Fig. 12C). In contrast, differently to what was observed in ALS mice, mKL levels in the spinal cord were unaltered (Fig. 12E).

We pooled and analyzed forelimb muscles together at 28 dpi, when muscle atrophy is prominent, and found that both sKL (0.12 ± 0.07) and mKL (0.3 ± 0.09) levels were reduced compared to the non-denervated forelimb (Fig. 12D, F). These alterations in the levels of KL suggest potential functions for this protein in the correct homeostasis of the spinal cord and skeletal muscles, which are disrupted in situations of neurodegeneration and atrophy.

1.2. Klotho protects motoneurons in an *in vitro* model of motoneuron degeneration

To characterize the direct effect of KL on MN survival we used an *in vitro* model of spinal cord organotypic cultures (SCOCs) subjected to acute glutamate-induced excitotoxicity. Viral vectors were employed to overexpress and assess the therapeutic potential of both sKL and mKL. The first step involved optimizing the infection using Adenoviral vectors coding for the green fluorescent protein (GFP).

After seeding and allowing the spinal cord slices to stabilize for one week, they were infected with Ad5-CMV-GFP vectors. These viral preparations were added to the media, which bathes the slices from underneath, at a final concentration of 10^6 or 10^8 PFU/mL. In parallel, vectors were directly administered as a single one-microliter droplet containing 10^6 or 10^8 total PFU onto the slice (Fig. 13A). The addition of Ad5 vectors to the media at 10^8 PFU/mL resulted in very poor transduction of the SCOCs (Fig. 13C), barely noticeable when diluted at 10^6 PFU/mL (image not shown). The addition of a one-microliter drop on top of the slice did not seem to swamp or alter the appearance of the appearance. The best transduction efficiency was observed when adding on the surface of the slice 10^8 PFU, the maximum amount possible at the working titers (Fig. 13B).

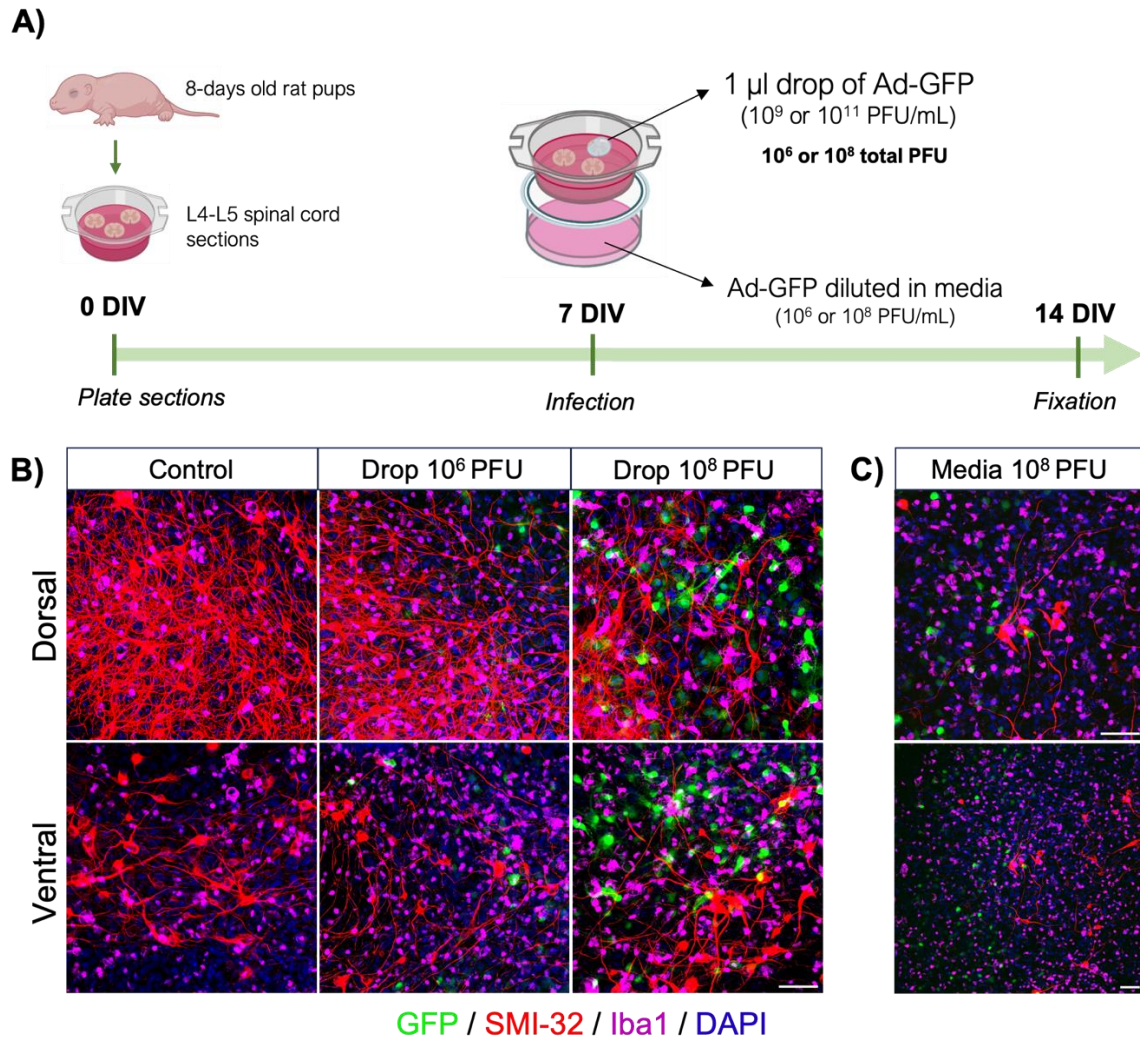


Figure 13. Establishment of the infection technique of SCOCs. (A) Experimental timeline for the culture preparation, infection with Ad5-GFP vectors, and fixation for posterior analyses. (B) Infection was performed either with 10^6 or 10^8 total PFUs on a one-microliter drop or directly added to the media at 10^6 or 10^8 IU/mL (C). Scale bar = 100 μ m.

Adenoviral vectors commonly show greater transduction efficiencies in cell cultures and primary dissociated cultures compared to AAV vectors. However, because these cultures are derived from animal explants, we sought to investigate whether AAV vectors would be better suited for transducing the diverse cell types present in the spinal cord. Employing the same droplet infection method, we conducted a comparison by infecting with 10^8 PFU of Ad5-CMV-GFP or with 10^{10} vg of AAV9-CMV-GFP vectors. The results revealed that AAV9 vectors exhibited higher transduction of cells in the SCOCs than Ad5 vectors at the titers used (Fig. 14C, D).

Notably, Ad5 vectors did not effectively transduce neuronal populations ($0.07 \pm 0.07\%$), while AAV9 vectors demonstrated a slight colocalization of GFP signal with the SMI-32 neuronal marker ($13.11 \pm 4.6\%$, Fig. 14E). Among the studied cell types, astrocytes labeled with GFAP exhibited the highest colocalization with the GFP signal (Fig. 14F).

AAV9 vectors ($17.15 \pm 6\%$) showed greater colocalization than Ad5 vectors ($8.4 \pm 2.7\%$), although the difference was not significant. It is important to note that for both vectors the percentage of transduced cells within each population varied significantly depending on the slice, ranging from 0 to almost 40% of the total cell subtype.

Microglia, immunostained for Iba1 (Fig. 14G), showed reluctance to transduction by Ad5 and AAV9 vectors ($0.52 \pm 0.3\%$ and $0.46 \pm 0.3\%$, respectively). Predominantly, transduction for both vectors occurred in unlabeled glial cells. The morphology of transduced cells pointed to oligodendrocytes, which are characterized by processes and a distinct branching pattern (Fig. 15B). However, labeling with the NG2 and O4 markers failed to stain oligodendrocytes in the SCOCs, requiring further studies to confirm the specific tropism to this cell type. Nonetheless, given that both sKL and mKL result in circulating proteins, robust transduction of neurons was not a prerequisite to studying the potential therapeutic effect of KL in these cultures.

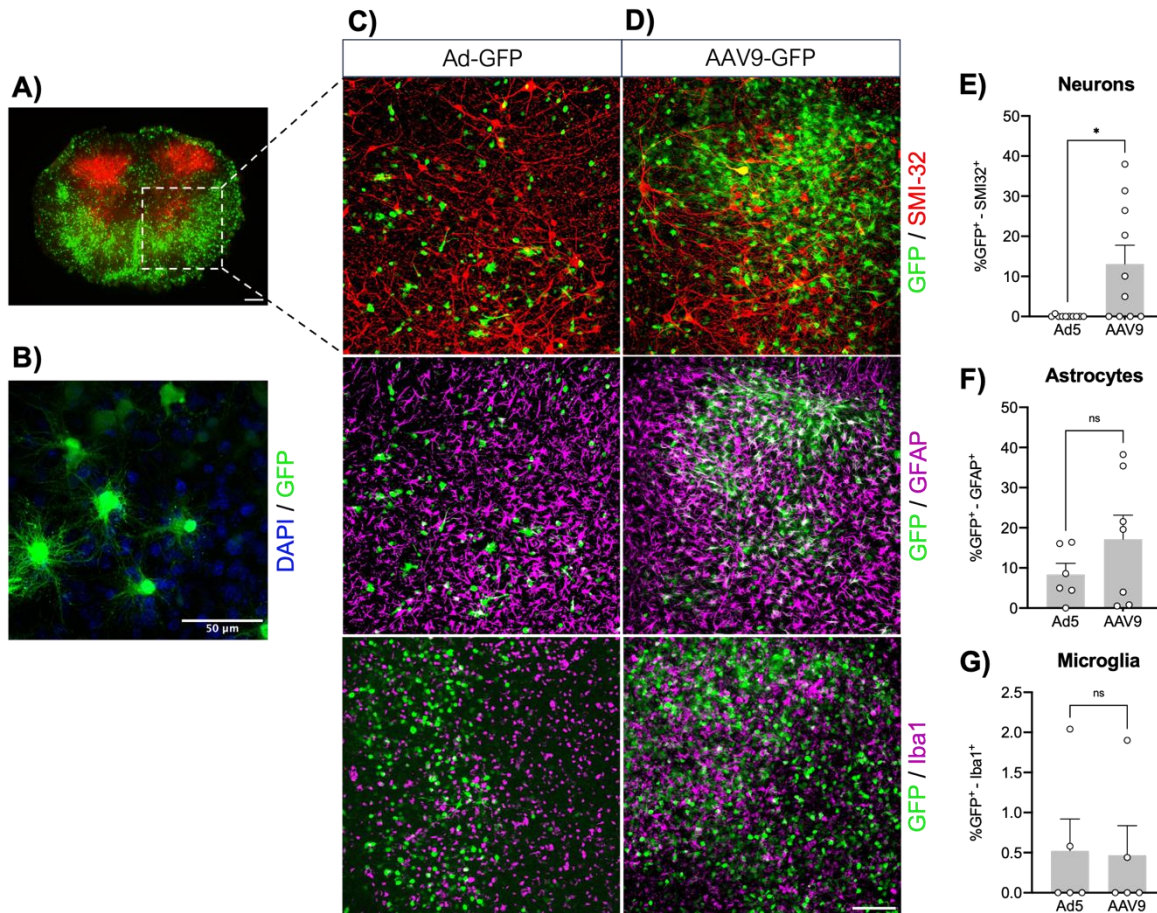


Figure 14. Comparison of Ad-GFP and AAV9-GFP vectors for the infection of SCOCs. Cultures were infected in parallel applying a droplet on top of the slice with either 10^8 IU of Ad5-CMV-GFP (C) or 10^{10} vg of AAV9-CMV-GFP (D). Image of an entire SCOC slice at low magnifications (A) and a high magnification image of the ventral horn showing the morphology of transduced cells (B). Quantification of the colocalization of GFP signal with SMI-32 (E), GFAP (F), and Iba1 (G) signal. Two-tailed Student's t-test, $*p < 0.05$. Data is shown as mean $\pm$ SEM ($n = 5-10$ slices). Unless specified, the scale bar is set to 100 μm .

Next, we continued with AAV vectors and implemented a similar protocol infecting the SCOCs with 10^{10} vg of AAV9-CMV-sKL, AAV9-CMV-mKL, or AAV9-CMV-Null to investigate if KL isoforms could act as neuroprotective agents. Control slices were treated with a drop of phosphate-buffered saline to examine whether viral vectors could alter culture homeostasis. Following the infection, the cultures were maintained for an extra week to allow for transgene expression, after which they were subjected to a 30-minute exposure to glutamate. Five days later, the spinal cord slices were collected for the assessment of KL neuroprotection (Fig. 15A).

The addition of glutamate significantly reduced the total number of SMI-32 positive cells in the ventral horn of the spinal cord (59.72 ± 5.9), compared to non-exposed control slices (100.35 ± 3.7). AAV9-Null treated slices showed comparable numbers of neurons (100.33 ± 4.8), which decreased similarly after the addition of glutamate (66.95 ± 4.7), indicating that the AAV9 viral particles per se do not interfere with neuronal survival (Fig. 15B, D).

The overexpression of sKL and mKL alone in SCOCs did not result in any significant change in the amount of positive SMI-32 cells in the slices (92.94 ± 4.6 and 94.26 ± 5.4 , respectively). However, after the excitotoxic insult, both sKL (99.26 ± 5.2) and mKL (101.4 ± 6.6) treated slices exhibited complete preservation of SMI-32+ neurons, preventing the neuronal loss observed in the control slices (Fig. 15B, D). This strongly indicates that both isoforms of KL can effectively protect neurons and, particularly, MNs from glutamate-induced excitotoxicity.

As for the quantification of KL-positive cells, we detected that AAV9-CMV-sKL transduced more cells (358.54 ± 26.3) compared to AAV9-CMV-mKL (276.83 ± 22.8) at the same dose (Fig. 15C, E), although this difference did not reach statistical significance ($p = 0.09$). This suggests that the vector encoding sKL has higher infectivity. Curiously, the counts of mKL-positive cells are significantly reduced in glutamate-treated slices, indicating degeneration of non-neuronal cells despite their expression of mKL, which differs from the outcome observed with sKL (Fig. 15E). Nevertheless, the levels achieved for both KL isoforms are still adequate to retain therapeutic efficacy on neurons.

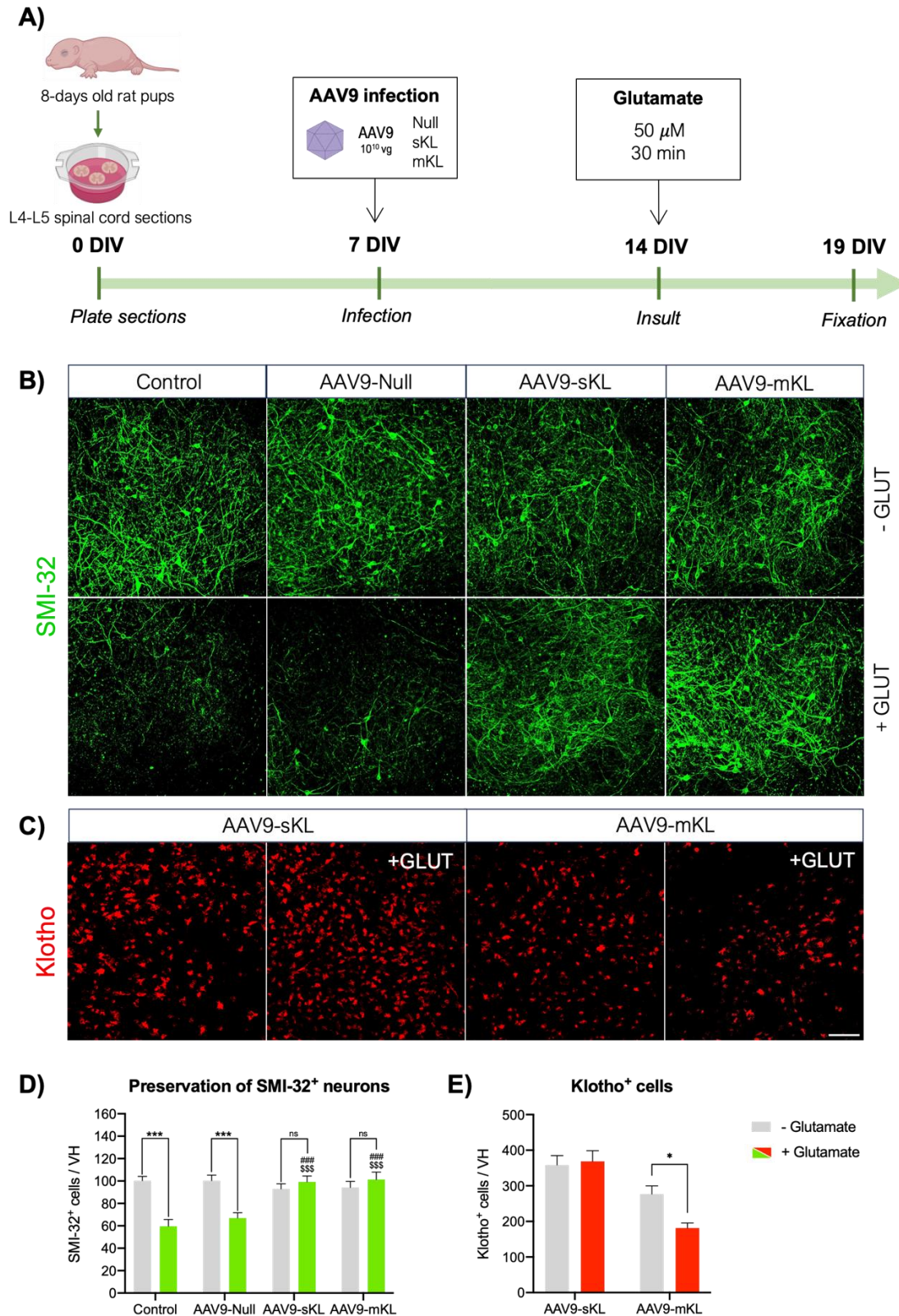


Figure 15. Klotho protects MNs from acute excitotoxic insult in spinal cord organotypic cultures (SCOCs). (A) Cultures were treated with AAV9 vectors to overexpress sKL, mKL, or a null sequence or non-treated, and exposed to excitotoxicity by glutamate. Representative microphotographs of MNs in the ventral horn of the slices labeled with the SMI-32 antibody (B) and of cells stained against Klotho (C) at 19 days in vitro (DIV). Scale bar = 100 μ m. Histogram graphs showing the number (mean $\pm$ SEM, n=17-25 hemisections per treatment) of SMI-32 (D) or of KL-positive cells (E) in the ventral horn of each spinal cord slice. Two-way ANOVA, * p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001; "##" indicates comparisons with the non-glutamate-exposed equivalent, "\$" with the control + glutamate group, and "#" with the AAV9-Null + glutamate group.

1.3. Overexpression of Klotho in the spinal cord of SOD1^{G93A} mice

Given the capacity of Klotho to safeguard MNs from excitotoxicity in the SCOCs, we proceeded to treat SOD1^{G93A} mice. Lumbar intrathecal injections were performed to deliver AAV9 vectors containing the secreted Klotho gene under the control of the ubiquitous CAG promoter to the spinal cord. Six-week-old SOD1^{G93A} mice were treated with a total of 2×10^{11} vg of AAV9-CAG-sKL vectors as a preventive strategy, considering the 2–3-week timeframe needed for AAV vectors to reach their maximum transgene expression levels (Fig. 16). An AAV9 with the CAG promoter and a null non-coding sequence (AAV9-CAG-Null) was employed to treat mock control animals. Intrathecal administration of AAV vectors can efficiently transduce the spinal cord following a gradient from the administration site, at the lumbar area, to the cervical segments. In addition, transduction is also present in the brain, with special evidence in the cortex. Moreover, AAV vectors mainly infect differentiated cells such as neurons, including MNs (Homs et al., 2014; Pagès et al., 2019).

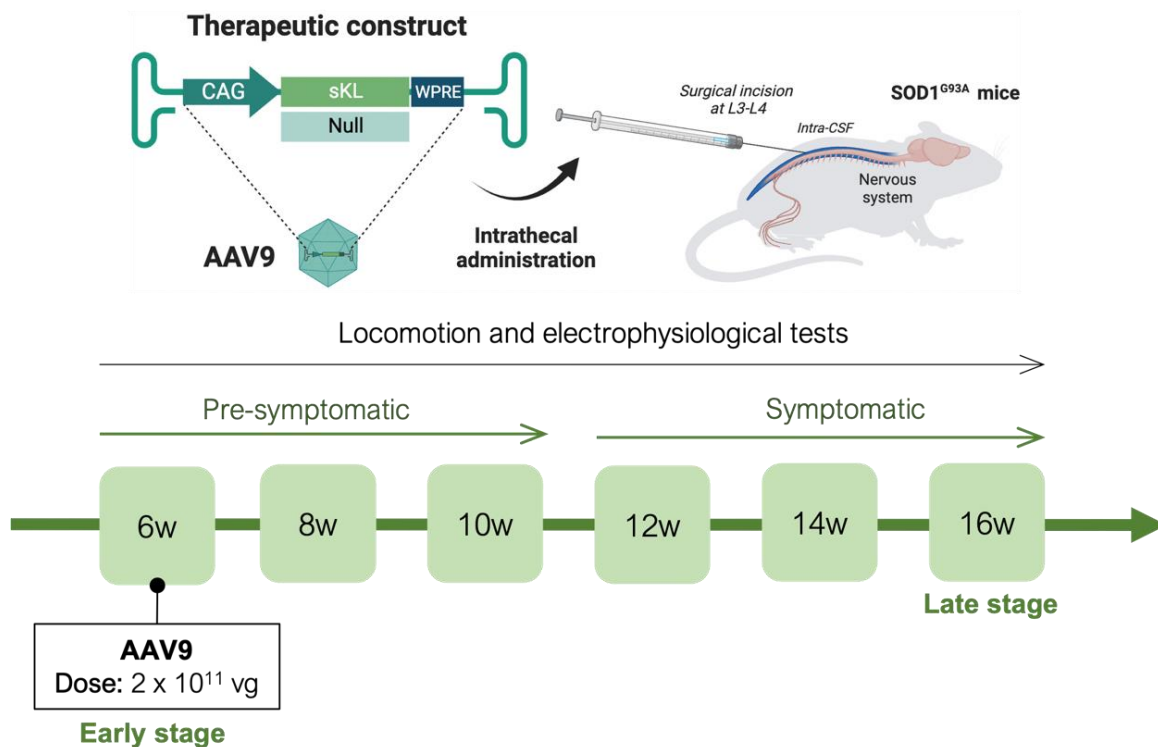


Figure 16. Study design for the administration and monitoring of AAV9-injected SOD1^{G93A} mice. Animals were intrathecally injected with 2×10^{11} vg of AAV9-CAG-sKL or null vectors at an early phase and monitored weekly by rotarod tests and at 8, 12, and 16 weeks of age by nerve conduction tests (females $n = 10$ WT, 13 SOD1 mock, 6 SOD1 sKL; males $n = 6$ WT, 9 SOD1 mock, 4 SOD1 sKL).

1.3.1. sKL partially preserves neuromuscular function in female SOD1^{G93A} mice

Electromyography tests were carried out at 8, 12, and 16 weeks of age to characterize the preservation of the neuromuscular function after the treatment. Klotho overexpression in the spinal cord only preserved the CMAP amplitude of the TA muscles (Fig. 17C) in the female cohort at 8 and 12 weeks of age (55.87 ± 3.7 mV and 47.47 ± 1.8 mV) compared to SOD1 mock-treated females (41.72 ± 3.4 mV and 29.7 ± 3.3 mV), while showing a trend at 16 weeks (25.58 ± 4.9 mV vs. 12.3 ± 1.9 mV). For the more distal plantar interossei (PL) muscles the treatment failed to preserve the CMAP amplitudes (Fig. 17A, B) in both sexes.

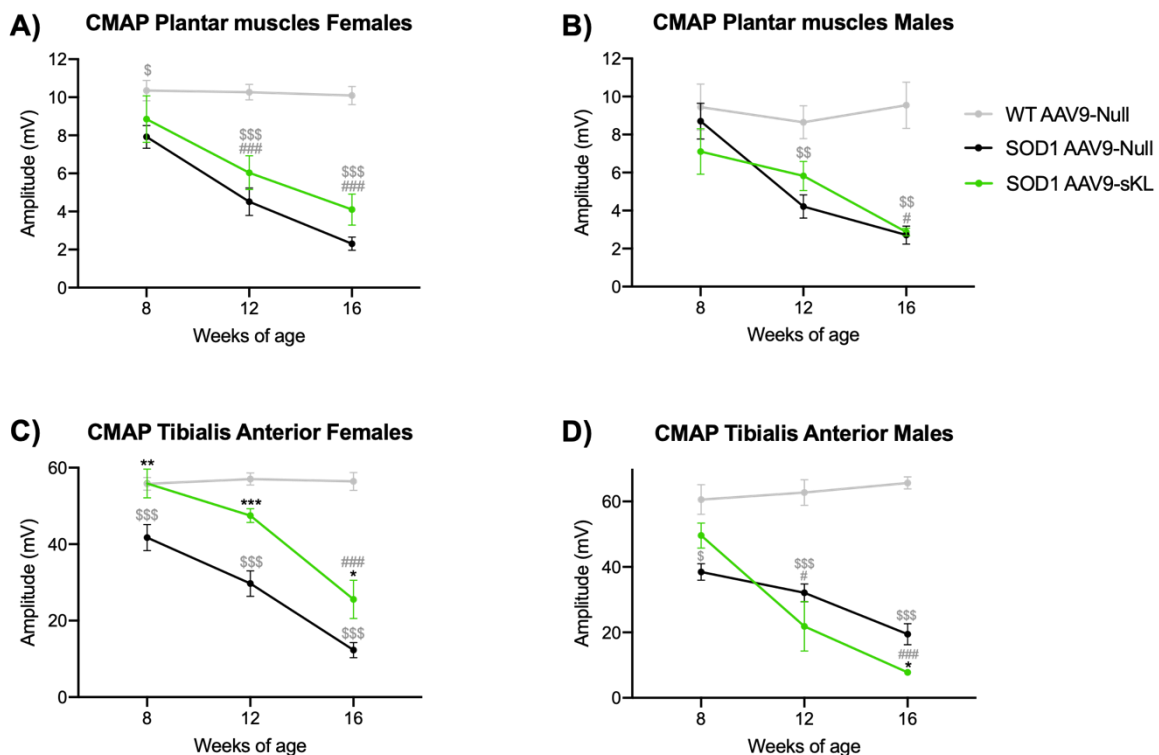


Figure 17. sKL overexpression in the spinal cord only preserves TA CMAP in females. CMAP amplitudes of the plantar interossei muscles (A, B) and of the tibialis anterior muscle (C, D) of female and male mice at the ages tested (Females n=10 WT, 13 SOD1 Null, 6 SOD1 sKL; Males n=6 WT, 9 SOD1 Null, 4 SOD1 sKL per group, two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; * SOD1 sKL relative to SOD1 Null and # relative to WT, \$ WT relative to SOD1 Null). Data is shown as mean $\pm$ SEM.

To evaluate the preservation of cortical MNs and the integrity of corticospinal and brainstem-spinal tracts we recorded the motor evoked potentials (MEP). MEPs were elicited by the subdermal electrical stimulation of the cortex and the response was recorded at the TA muscle. In mice, MEP recordings exhibit two components: an initial component with shorter latency, believed to be brainstem-evoked, and a subsequent later component that is cortically elicited. The amplitude of the TA MEPs was preserved until 12 weeks of age in sKL-treated SOD1^{G93A} females (Fig. 18A, C) for the first (14.35 ± 1.3 vs. 4.44 ± 0.7) and the second component (10.47 ± 1 vs. 2.8 ± 0.6), and significantly higher than in untreated mice.

At the end stage of the disease (16 weeks) it also tended to be higher than in female SOD1^{G93A} mock control animals. On the contrary, in treated males, the overexpression of sKL did not yield any positive outcomes concerning MEP amplitudes (Fig. 18B, C), as for the CMAP amplitude recorded in the motor nerve conduction tests (Fig. 18).

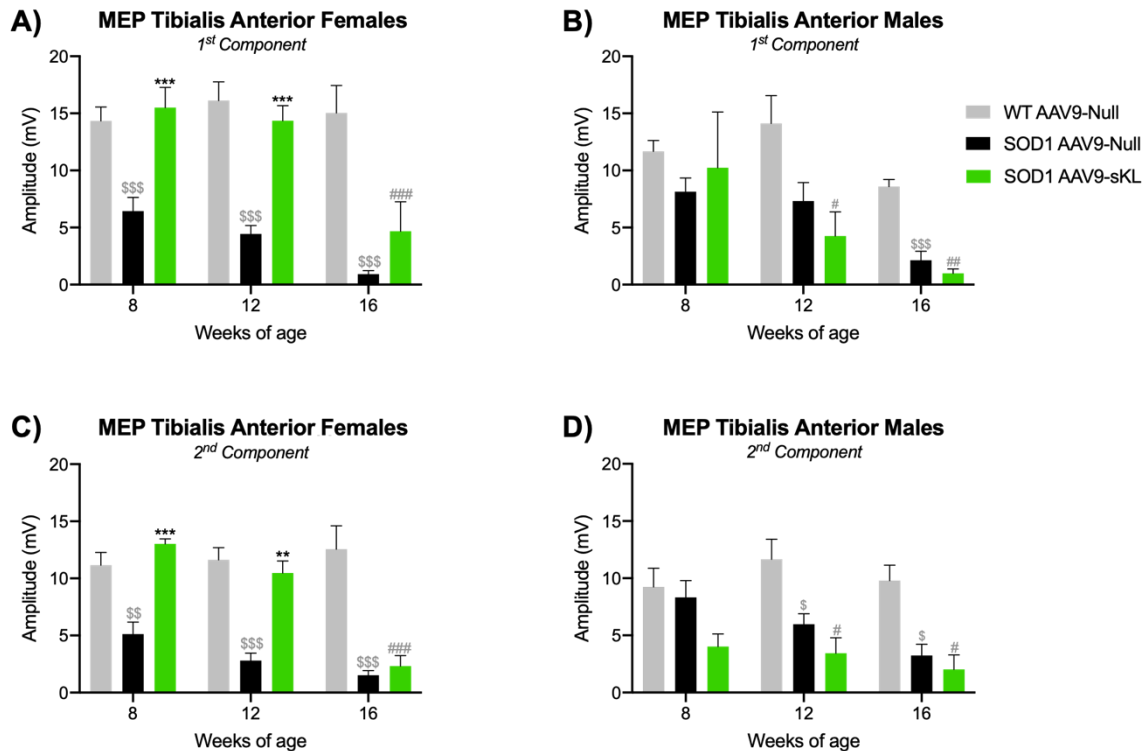


Figure 18. The amplitude of the MEP is preserved only in s-KL-treated SOD1^{G93A} female mice. MEP amplitudes for both components recorded at the tibialis anterior muscle of female (A, C) and male (B, D) mice at the ages tested (Females n=10 WT, 13 SOD1 Null, 6 SOD1 sKL; Males n=6 WT, 9 SOD1 Null, 4 SOD1 sKL per group, two-way ANOVA, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001; * SOD1 sKL relative to SOD1 Null and # relative to WT, \$ WT relative to SOD1 Null). Data is shown as mean ± SEM.

1.3.2. sKL preserves motor function in SOD1^{G93A} mice but fails to maintain body weight and muscle mass

Body weight and rotarod performance were monitored as functional outcomes of the treatment, as well as the muscle mass at the time of sacrifice. Both female and male SOD1^{G93A} treated mice had the same evolution of body weight through the progression of the disease as untreated mice (Fig. 19A, B). However, despite the lack of neuromuscular function improvement in treated males, both females and males exhibited a better performance in the rotarod test than SOD1^{G93A} mice injected with AAV9-Null vectors (Fig. 19C). The gastrocnemius muscle was markedly atrophied at 16 weeks in SOD1^{G93A} mice, with reduced mass in both females (77.3 ± 8.1 mg) and males (86.6 ± 6.1 mg) compared to the WT mock group (138.35 ± 4.2 and 178.72 ± 9.2 mg, respectively).

The treatment showed a slight, although not significant preservation (96.2 ± 10 mg in females and 100.88 ± 9.5 mg in males) of the gastrocnemius muscle weight.

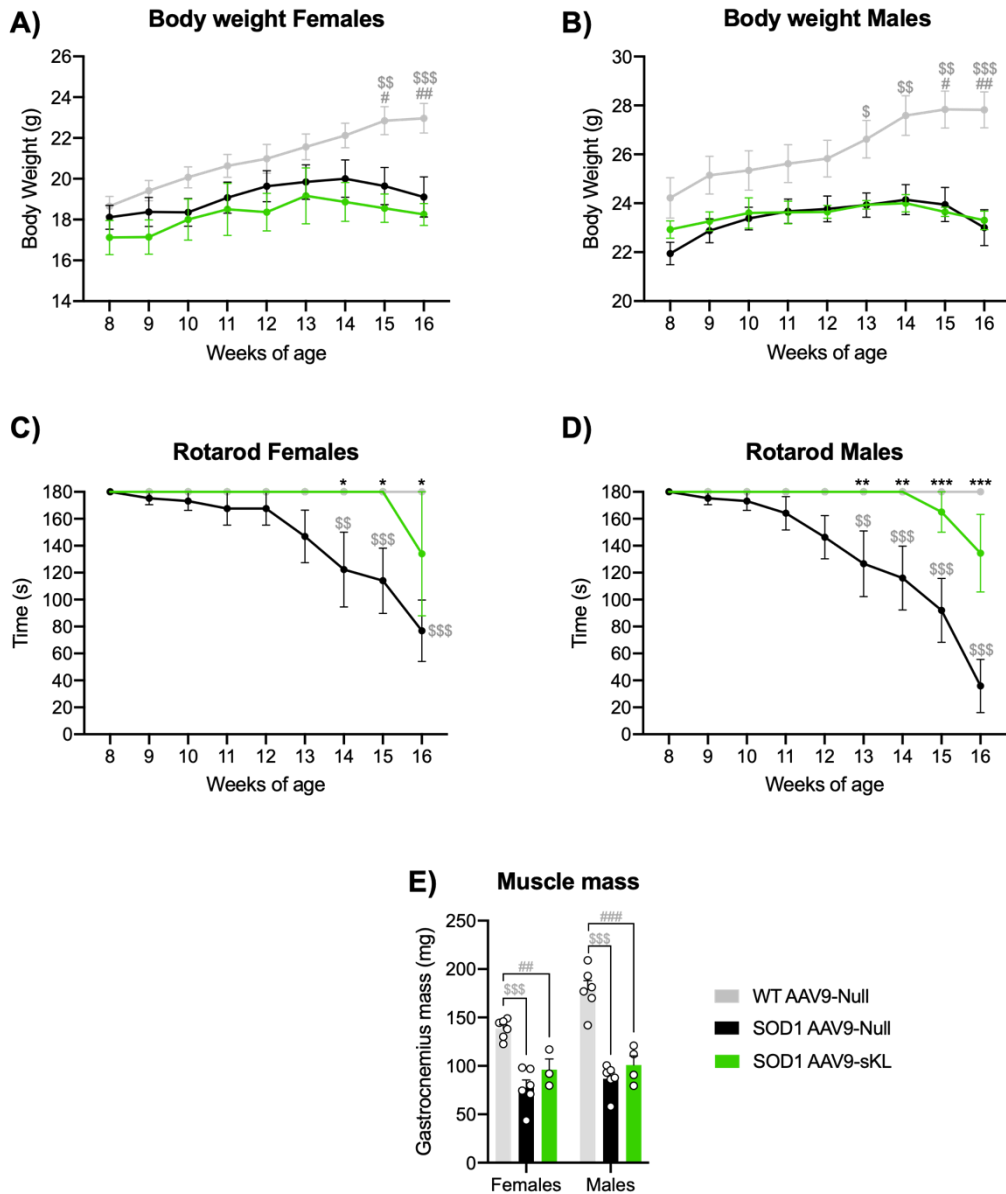


Figure 19. Overexpression of sKL in the spinal cord does not correct body weight and muscle mass loss but improves rotarod performance. (A, B) Body weight progression and (C, D) rotating rot performance of both sexes pooled together (Two-way ANOVA). (E) Gastrocnemius muscle mass at 16 weeks. One-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*), vs. WT Null (#), WT vs. SOD1 Null (\$). Data presented as mean $\pm$ SEM.

Chapter II:

Targeting skeletal muscle-specific overexpression
of Klotho with systemic AAV8 vectors

2.1. Overexpression of Klotho in muscles with AAV8 vectors

In previous research, we successfully confined therapeutic gene expression to the muscles of SOD1^{G93A} mice, resulting in superior outcomes compared to intrathecal delivery (Mòdol-Caballero et al., 2020b; 2021a; 2021b). To overexpress Klotho in muscles we intravenously administered AAV8 vectors containing the secreted mouse Klotho cDNA regulated by the human desmin promoter. With this formerly validated systemic administration of AAV8 vectors and the use of the muscle-specific promoter, we achieved widespread and restricted expression of sKL to striated muscles. In a first study, we treated 6-week-old SOD1^{G93A} mice with 1.8×10^{14} vg/kg or 3×10^{14} vg/kg of AAV8-Des-sKL vectors via tail vein injection as a preventive strategy. Null vectors with the desmin promoter and a stuffer sequence (AAV8-Des-Null) were administered to mock control mice. Corresponding control groups of wild-type and SOD1 mice received a dose of 1.8×10^{14} vg/kg AAV8-Des-Null. To evaluate the effectiveness of this therapeutic approach we focused on treating female SOD1^{G93A} mice. Mice were followed as described in Fig. 20.

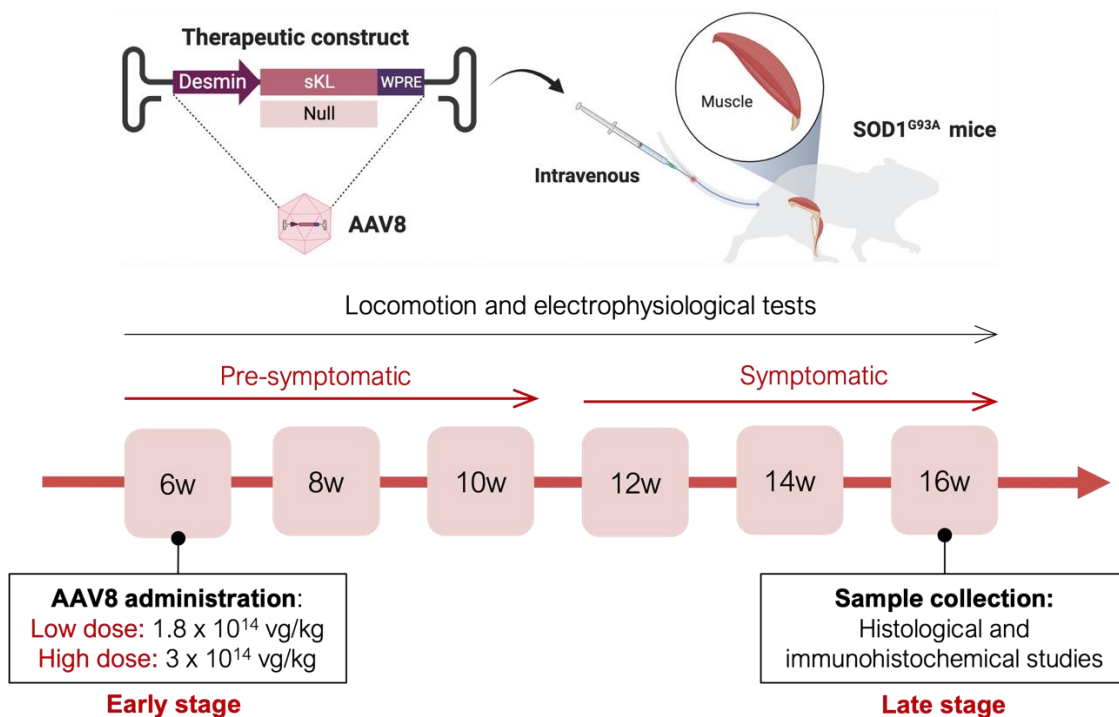


Figure 20. Experimental design for the follow-up of AAV8-injected SOD1^{G93A} mice. Female mice were administered systemically with 1.8×10^{14} vg/kg or 3×10^{14} vg/kg of AAV8-Des-sKL, or with null vectors at 1.8×10^{14} vg/kg at an early stage and followed up weekly by rotarod tests and at 8, 12 and 16 weeks of age by nerve conduction tests (n = 9 WT, 12 SOD1 Null, 8 SOD1 sKL LD, 15 SOD1 sKL HD).

2.2. AAV8-Des-sKL preserves neuromuscular function in female SOD1^{G93A} mice

The electrophysiological evaluation revealed that the treatment in female SOD1^{G93A} mice at the low dose did not reduce the progressive decline of the CMAP amplitude of the hindlimb muscles in comparison to animals treated with the null vector (Fig. 21A, C). Similarly, MEP amplitudes were not preserved by the treatment (Fig 22A, B). Although there was a tendency for preservation in treated female mice at 8 weeks of age, from 12 weeks onwards the amplitude dropped to levels comparable to the SOD1 mock group.

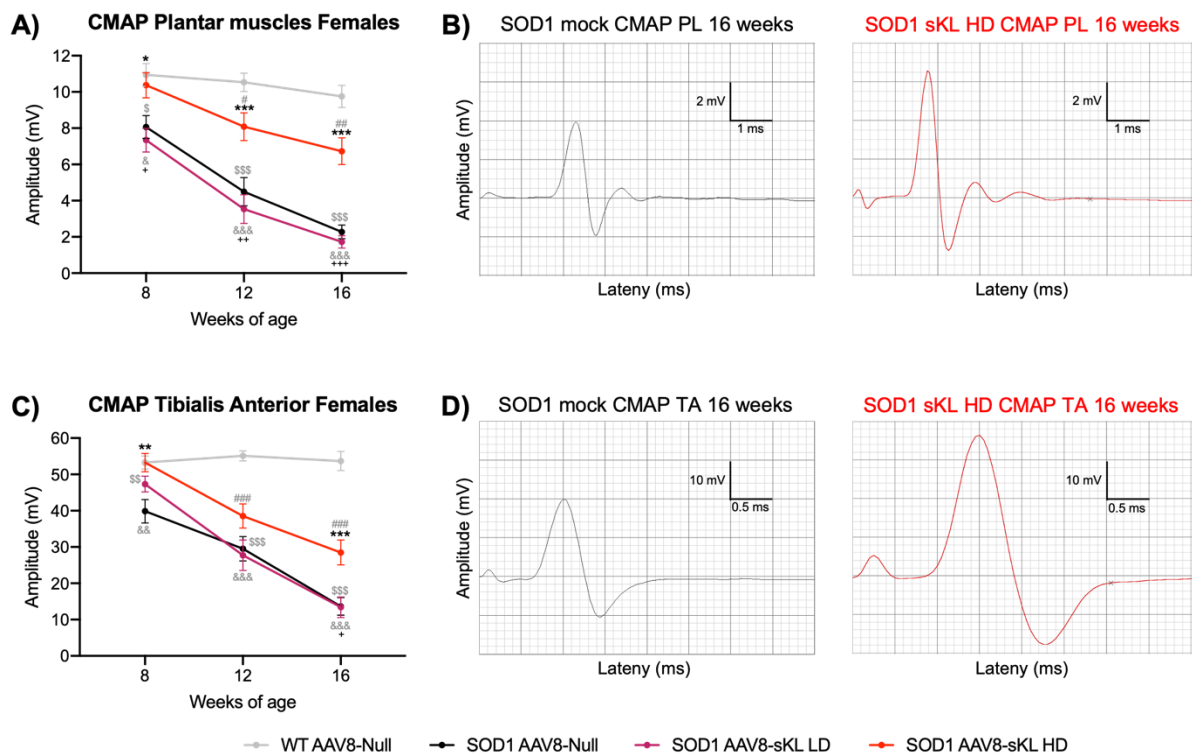


Figure 21. KL overexpression mediated by the high AAV8 dose leads to CMAP preservation of hindlimb muscles in SOD1^{G93A} female mice. CMAP amplitudes of the PL (A, B) and TA muscles (C, D) with a representative recording for the SOD1 Null and sKL mice. Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*), vs. WT Null (#) or SOD1 sKL LD (+); WT vs. SOD1 Null (\$) or vs. SOD1 sKL LD (&). Data is shown as mean $\pm$ SEM.

With the high dose, we examined the highest systemic AAV viral titers tested in clinical settings (3×10^{14} vg/kg). We aimed to attain the highest Klotho expression possible within muscles using AAV8 vectors. Contrary to the low dose, the high dose of AAV8-Des-sKL demonstrated that the amplitudes of the CMAP were notably preserved compared to SOD1^{G93A} mock mice. This preservation was significant at the three-time points examined for the PL muscles (Fig 21A, B) and at 8 and 16 weeks for the TA muscle (Fig 21C, D). At the late stage of the disease, CMAP amplitudes of the PL and TA muscles in treated female mice (6.73 ± 0.74 and 28.49 ± 3.43 respectively) more than doubled the values observed in SOD1^{G93A} controls (2.27 ± 0.37 and 13.65 ± 2.43).

Interestingly, higher levels of muscle-secreted KL were able to uphold central motor pathways at the earlier stages, as demonstrated by the significant maintenance of MEP amplitudes at 8 and 12 weeks (Fig. 22A-D). The first MEP component also showed an increased amplitude at 16 weeks of age, while the second MEP component displayed a trend when compared to mock counterparts.

Despite not yielding significant differences, the MEP/CMAP ratio suggests that corticospinal connections are preserved for at least 12 weeks and that this preservation is lost by the time animals reach the end stage (Fig. 22E, F). This ratio assesses the relative contribution of UMN involvement in the overall motor pathway, as the recording of MEP in the muscle depends on LMN synapsis.

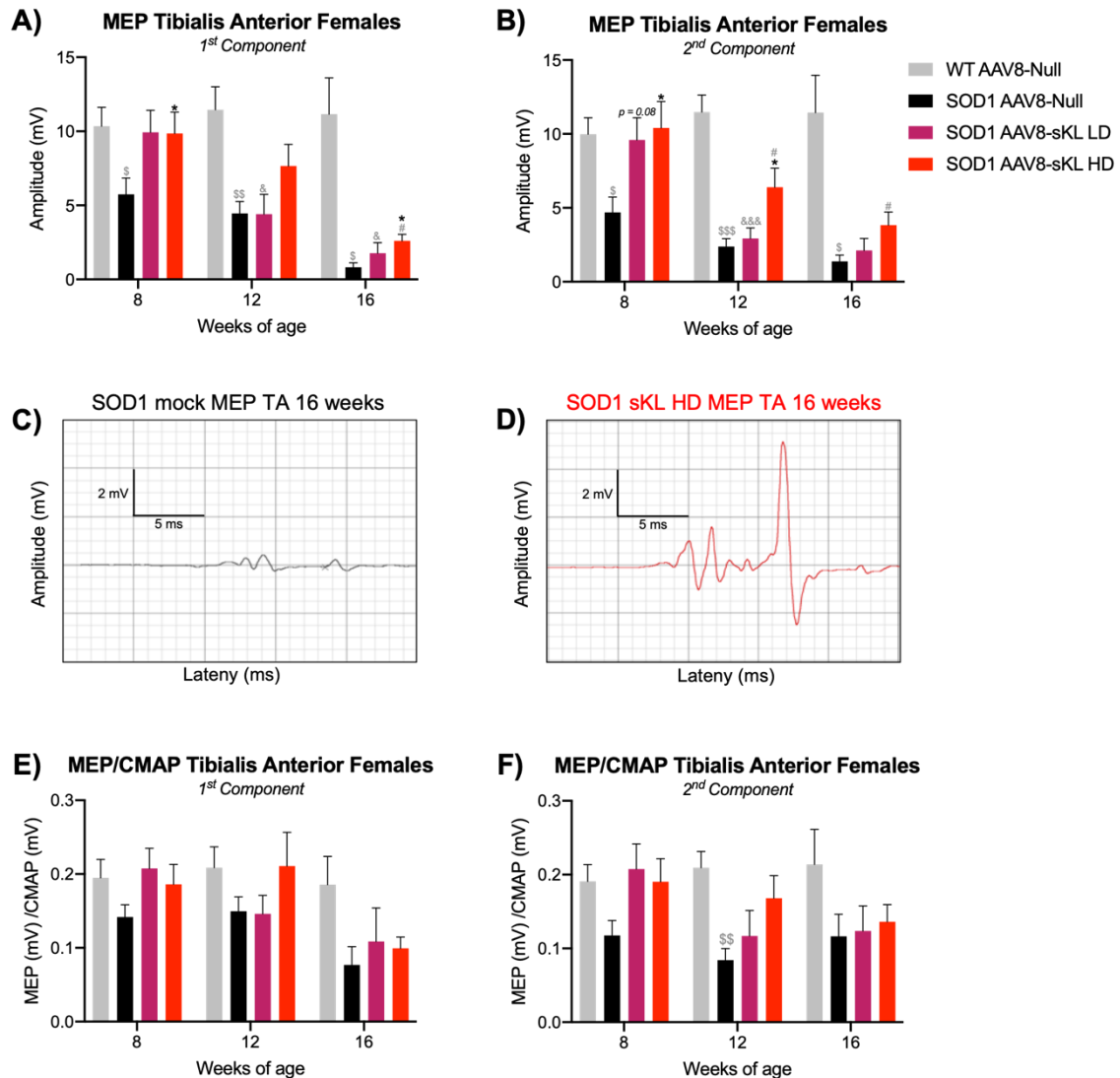


Figure 22. SOD1^{G93A} female mice treated with the higher titer of AAV8-Des-sKL display preserved corticospinal connections. Mean peak MEP amplitudes for the first (A) and second (B) components observed during TA recordings (C, D). MEP/CMAP amplitude ratio for both components (E, F). Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL HD relative to SOD1 Null (*) or to WT Null (#), WT relative to SOD1 Null (\$), SOD1 sKL LD relative to SOD1 Null (&). Data is depicted as mean $\pm$ SEM.

2.3. AAV8-Des-sKL boosts overall function in female SOD1^{G93A} mice

Secretion of KL in the muscle was able to produce functional motor improvements in SOD1^{G93A} females compared to null-injected mice, particularly at the higher dose. The low-dose therapy failed to mitigate the reduction in body weight gain and the characteristic body weight loss observed in SOD1^{G93A} mice during the late stages of the disease (Fig 23. A). Surprisingly, body weight in high-dose-treated mice remained below that of SOD1 mock animals, that received injections of AAV8-Des-Null at the lower dose of 1.8×10^{14} vg/kg.

The locomotion of the animals was evaluated by the rotarod test. Low dose AAV8-treated mice exhibited a slight improvement in rotarod performance at the 14th and 15th week compared to SOD1 Null controls but was lost when the animals reached 16 weeks. In the high dose group, motor performance and coordination were significantly improved to end-stage (Fig. 23B). The clinical disease onset, defined as the timepoint when the animals first fall off the rotarod before the 180 seconds set time, was delayed in sKL-treated animals to 15 weeks of age (Fig. 23C). At 16 weeks, only $35.7 \pm 12.8\%$ of the high dose treated SOD1^{G93A} mice had motor deficits in the rotarod test, whereas $75 \pm 12.5\%$ of the SOD1 mock animals fell before the set time.

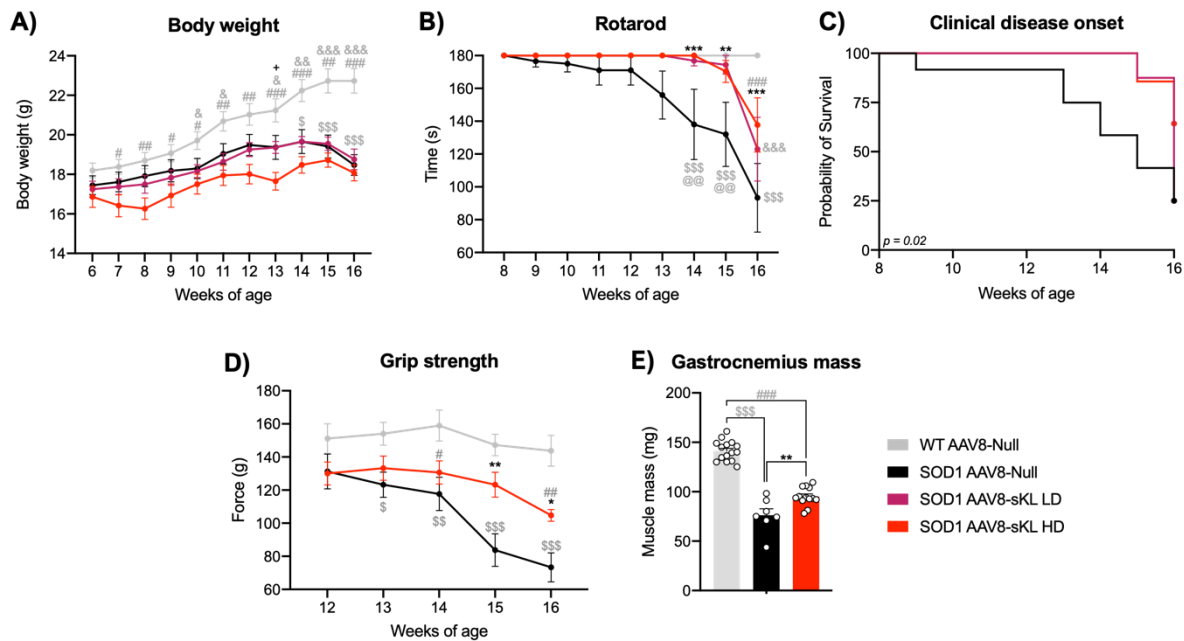


Figure 23. AAV8-Des-sKL gene therapy at the higher dose results in general functional improvements and a delay in the onset of the disease. KL overexpression did not improve body weight progression curves (A), but it enhanced rotarod performance (B) and grip strength (D) of female SOD1^{G93A} mice in the high dose group (Two-way ANOVA test). (C) The clinical onset of disease was significantly delayed (Mantel-Cox test, $p = 0.02$), and the mass of the gastrocnemius muscle preserved (One-way ANOVA test) (E). Data represented as mean $\pm$ SEM and Tukey's multiple comparisons test are as follows, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*), vs. WT Null (#) or SOD1 sKL LD (+); WT vs. SOD1 Null (\$) or vs. SOD1 sKL LD (&); SOD1 sKL LD vs. SOD1 sKL Null (@).

Animal strength was evaluated using the grip strength test in the high-dose group, which involved recording the maximum pulling force exerted by all four limbs on a grid. SOD1^{G93A} mice had lower grip strength than WT mice, with a marked decline after the 14th week, a decline that was significantly mitigated by the sKL treatment (Fig. 23D). These motor improvements were associated with the postmortem preservation of the GM muscle mass at 16 weeks of age despite showing an overall reduced body weight (Fig. 23E). Overall, applying AAV8 vectors at the dose of 1.8×10^{14} vg/kg to achieve muscle-specific sKL expression did not lead to improvements in the neuromuscular status of SOD1^{G93A} mice, despite a slight improvement in the motor function was observed. On the contrary, SOD1^{G93A} mice treated at 3×10^{14} vg/kg experienced preservation in neuromuscular function, locomotion, and strength.

2.4. Muscular secretion of KL preserves MNs and NMJs, and decreases glial reactivity

To understand the functional benefits observed in high dose treated SOD1^{G93A} female mice we performed histological analysis of the spinal cord and GM muscle at 16 weeks of age to quantify spinal MNs and the innervation of NMJs. Importantly, the results showed that KL overexpression preserved the number of MNs in the lumbar spinal cord (13.74 ± 1.5 , mean number of MN in ventral horns $\pm$ SEM) compared to SOD1^{G93A} mock mice (8.62 ± 0.69), in line with our electrophysiological findings. The increased CMAP in the GM muscle at 16 weeks was further confirmed by the NMJ analysis, as gene therapy significantly preserved the number of occupied motor end plates (75.2 ± 5.6) in comparison to the mock SOD1^{G93A} group (40.56 ± 4.6).

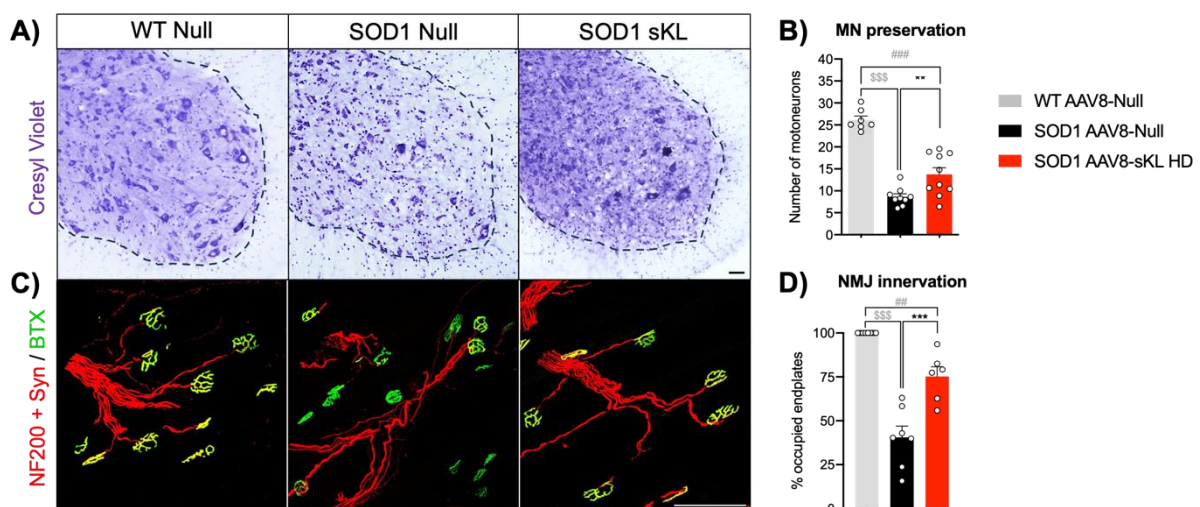


Figure 24. Muscle-specific KL overexpression protects spinal MNs and their synaptic connections to muscles in SOD1^{G93A} female mice. (A, B) The therapy protects the cresyl violet-stained neurons located in the ventral horn of the spinal cord at the L4-L5 level. This translates to a higher percentage of innervated NMJ at the gastrocnemius muscle (C, D). One-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar = 100 μ m. Mean $\pm$ SEM.

A hallmark of ALS recapitulated in SOD1^{G93A} mice is a strong inflammation of the spinal cord. We assessed microgliosis with Iba1 immunofluorescence, which was considerably reduced in treated mice (Fig. 25A, B). When we examined astrocyte reactivity with GFAP labeling, the levels in treated mice closely resembled those observed in the wild type (Fig. 25C, D). Furthermore, vimentin, a general marker for fibrosis and inflammation expressed by fibroblasts and cells of immune lineage, exhibited a significant downregulation after treatment (Fig. 25E, F). These results indicate that muscle-delivered Klotho is protecting the spinal cord from neuroglial reactivity.

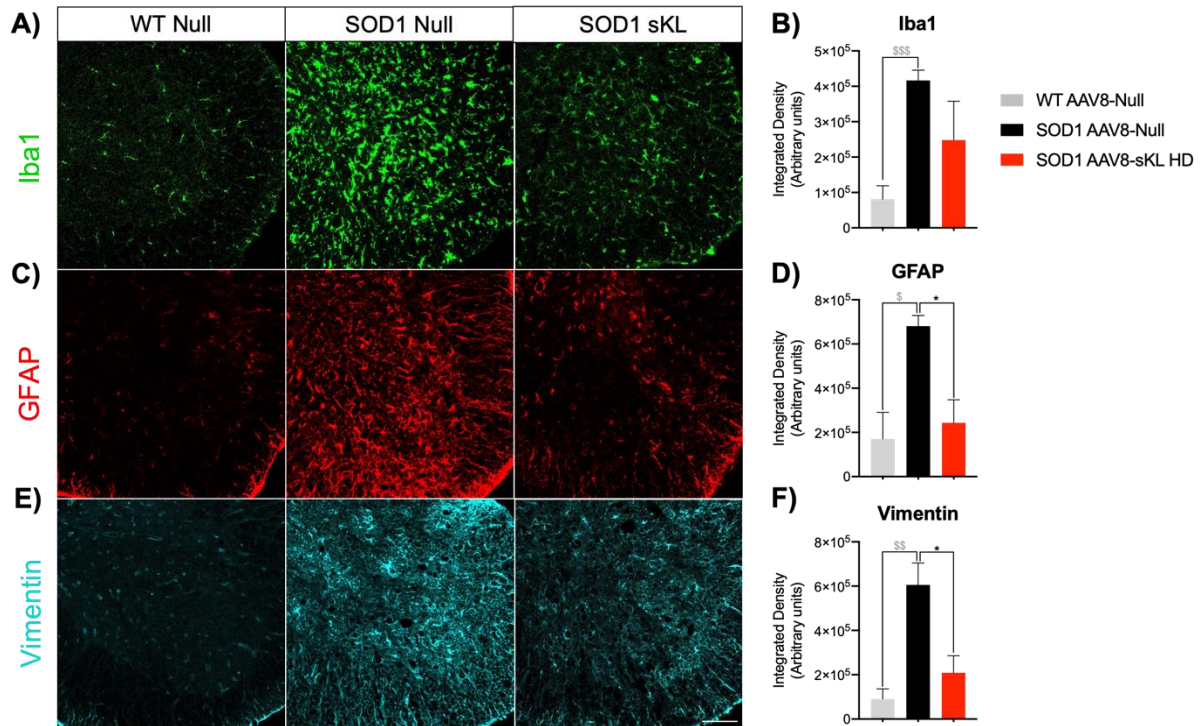


Figure 25. Klotho secretion from muscles protects the spinal cord from neuroinflammatory insults in ALS. Representative images of activated microglia labeled against Iba1 (A), activated astrocytes detected with GFAP (C), and the general inflammatory marker vimentin (E). Quantitative analysis of the inflammatory markers reveals a prominent downregulation in treated SOD1 female mice. (B, D, F). One-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar = 100 μm . Data plotted as mean $\pm$ SEM.

Chapter III:

Enhanced Klotho delivery to muscles with the
novel myotropic MyoAAV vector

3.1. MyoAAV-Des-sKL therapeutic dose-finding study

Given the substantial doses of AAV8-Desmin-sKL vectors needed to reach therapeutic effects in SOD1^{G93A} female mice, we explored the highly muscle-tropic MyoAAV vector (Tabebordbar et al., 2021). We aimed to find a more efficient AAV that would allow for dose sparing. The systemic administration of extremely high AAV doses to patients poses a significant manufacturing challenge, making our initial AAV8-Des-sKL therapeutic approach impractical. More importantly, such high viral doses have been associated with adverse effects in patients during clinical trials (Ertl, 2022).

We conducted a dose-escalating study with the MyoAAV vectors to achieve at least comparable benefits to those observed with AAV8 vectors. Three different doses (2×10^{12} , 8×10^{12} , or 1.6×10^{13} vg/kg) of the previously validated construct encapsulated into MyoAAV capsids were intravenously administered to SOD1^{G93A} mice at 6 weeks of age. Therapeutic efficacy was evaluated by nerve conduction tests at 8, 12, and 16 weeks. Wild-type and SOD1^{G93A} mock control animals were treated in parallel with MyoAAV-Des-Null vectors at the highest dose (Fig. 26).

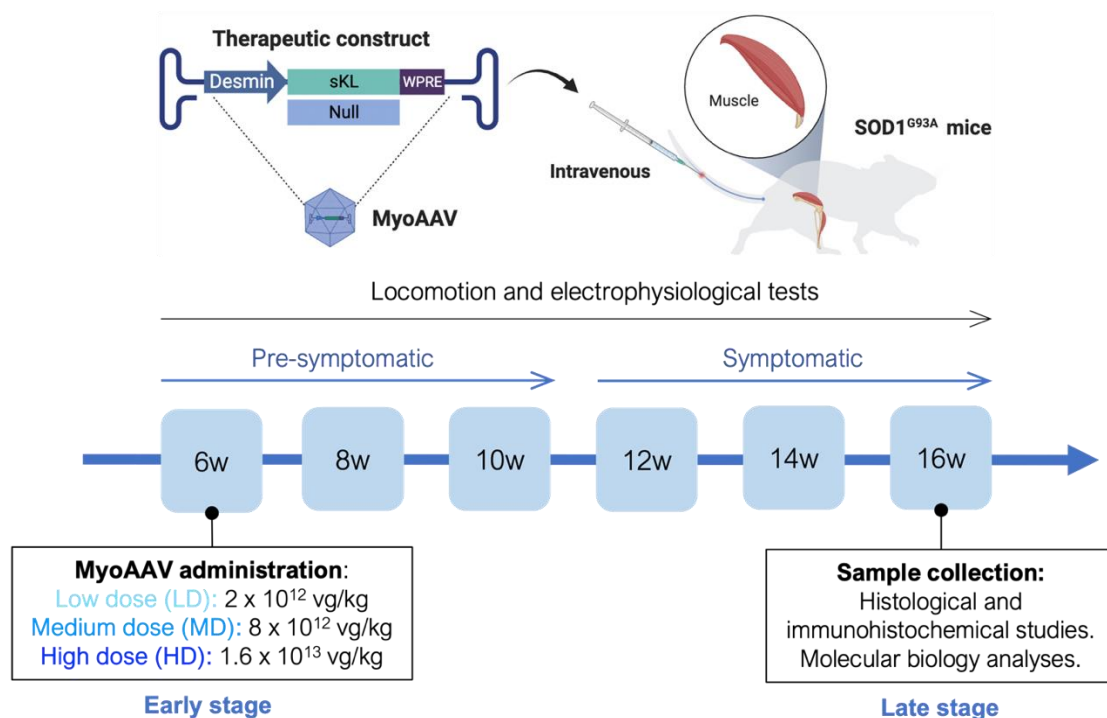


Figure 26. MyoAAV-Des-sKL dose-escalating study in SOD1^{G93A} mice. Animals were systemically administered at 6 weeks of age at three doses of MyoAAV-Des-sKL: 2×10^{12} , 8×10^{12} or 1.6×10^{13} vg/kg. Null vectors were injected at the highest dose. Therapeutic efficacy was assessed by electrophysiological tests at 8, 12, and 16 weeks of age. (n = 24 SOD1 Null, 5 SOD1 sKL LD; 9 SOD1 sKL MD and 16 SOD1 sKL HD).

The electrophysiological assessment revealed that systemic administration of MyoAAV-Des-sKL vectors was able to preserve the neuromuscular function, demonstrating a dose-response effect for the CMAP potentials in the muscles tested (Fig. 27A, B). For the highest dose of MyoAAV vectors, the PL and TA CMAP amplitude at 16 weeks (6.34 ± 0.4 and 33.64 ± 2.5 mV, respectively) was comparable to the preservation obtained with 3×10^{14} vg/kg of AAV8 vectors (6.73 ± 0.7 and 28.49 ± 3.4 mV, respectively) (see Fig. 21). The MEP potentials displayed a less clear dose-dependent response (Fig. 27C, D). However, animals treated at the highest dose showed slightly higher preservation in the MEP amplitude for the first (5.72 ± 1.6 mV) and second components (3.9 ± 0.6 mV) compared to AAV8-treated SOD1^{G93A} mice (2.6 ± 0.4 and 3.83 ± 0.8 mV, respectively) (see Fig. 22). Systemic administration of MyoAAV-Des-sKL at 1.6×10^{13} vg/kg produced outcomes comparable to those of AAV8 vectors at 3×10^{14} vg/kg, representing an 18.75-fold reduction in the dosage. Therefore, this dose was selected for subsequent studies.

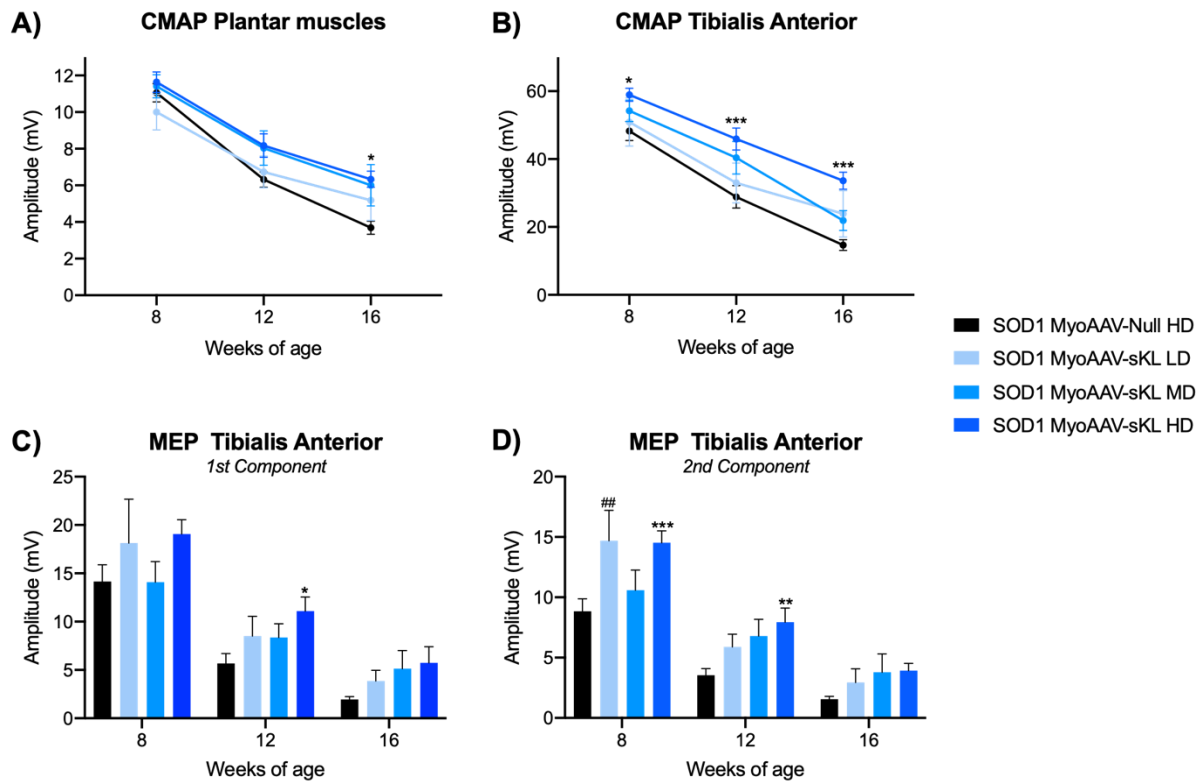


Figure 27. Assessment of MyoAAV-Des-sKL therapeutic doses by nerve conduction tests. CMAP amplitudes of the PL and TA muscles in the different dose-treated groups (A, B). MEP amplitudes for both components recorded at the TA muscle comparing administered doses (C, D). Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 Null relative to SOD1 sKL LD (#) or HD (*). Data plotted as mean $\pm$ SEM.

3.2. MyoAAV-mediated overexpression of sKL before disease onset

3.2.1. MyoAAV-Des-sKL preserves neuromuscular function in SOD1^{G93A} mice

To provide additional support for the functional improvement resulting from MyoAAV-mediated sKL overexpression in SOD1^{G93A} mice, more animals were incorporated in the study and treated with the dose of 1.6×10^{13} vg/kg. In parallel, to assess the safety profile of sKL muscle-restricted overexpression, we included a group of wild-type animals administered with MyoAAV-Des-sKL in the same conditions. Wild-type and SOD1^{G93A} mouse control groups received injections of MyoAAV vectors containing a non-coding stuffer sequence of approximately the same size as sKL. The analyses were conducted based on sex, and the follow-up procedures as outlined in Fig. 26.

In females, electrophysiological results (Fig. 28A-F) showed a significant preservation of the PL (7.39 ± 0.3 mV) and TA (43.26 ± 2.3 mV) CMAP amplitudes already by 12 weeks of age compared to SOD1^{G93A} Null-treated mice (5.7 ± 0.4 and 32.9 ± 3 mV, correspondingly). At 16 weeks of age, the CMAP amplitude for the PL (6.13 ± 0.3 mV) and TA (31.7 ± 1.8 mV) muscles almost doubled the values of the mock-treated animals (3.4 ± 0.3 and 17.2 ± 1.7 mV, respectively).

Male SOD1^{G93A} mice present a more rapidly advancing loss of neuromuscular function than females (Fig. 29A-F), with a significant reduction in the CMAP amplitude of the TA muscle (43 ± 3.8 mV) at 8 weeks compared to WT mice (62.47 ± 1.6 mV). Treatment preserved the CMAP amplitude at this earlier stage (56.7 ± 2.3 mV), as well as at 12 (33.8 ± 2.7 mV) and 16 weeks (21.86 ± 2.3 mV) compared to SOD1 mock animals (22.74 ± 3.4 and 12.36 ± 1.64 mV, respectively). These results indicate that the treatment significantly preserves the functional innervation of the hindlimb muscles. Wild-type animals administered with MyoAAV-Des-sKL showed no substantial differences in CMAP amplitudes compared to the wild-type group injected with the null vector.

The amplitude of the MEPs in treated females tended to be higher for both components compared to SOD1^{G93A} mock-treated controls (Fig. 28G-I and L), suggesting that sKL secretion from muscles preserved the descending corticospinal tracts. Specifically, at 12 weeks, the amplitude was significantly higher for the first (10.4 ± 0.9 mV) and the second component (8.4 ± 0.8 mV) in contrast to mock-treated counterparts (6 ± 0.8 and 4.3 ± 0.6 mV). When statistically comparing only treated and mock SOD1 animals, significant preservation was observed at the three ages tested. The MEP/CMAP ratio indicates that this preservation occurs independently of the maintenance of the second-order MN, which is not evident at the end stage (Figure 28J-K). Curiously, female WT mice with muscle overexpression of sKL exhibited modestly elevated amplitudes for the first component of the MEP in comparison to WT mock mice.

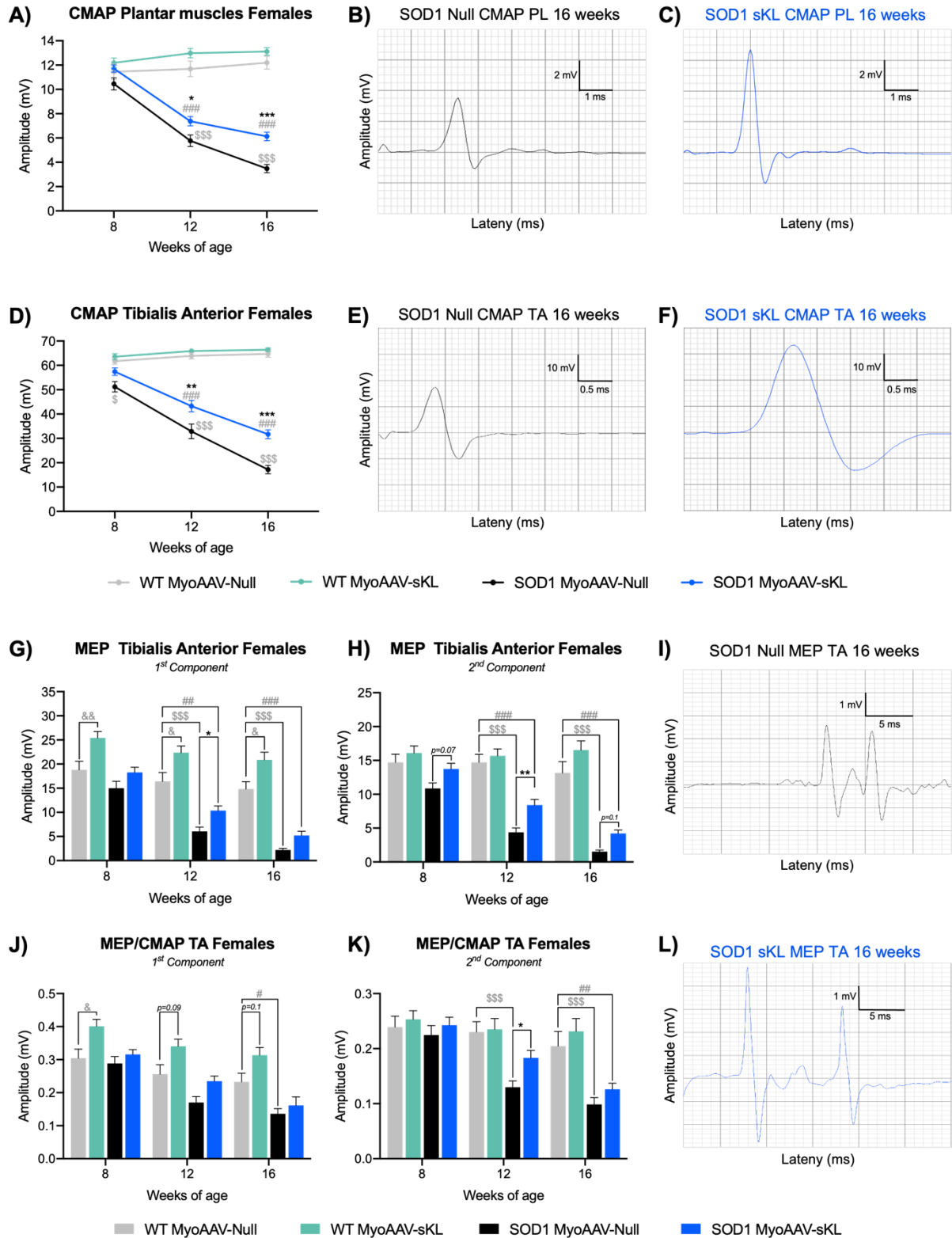


Figure 28. MyoAAV-mediated muscle overexpression of sKL promotes functional improvement in SOD1^{G93A} female mice. CMAP amplitudes of the PL (A) and TA muscles (D) with a representative recording at 16 weeks for SOD1 Null female mice (B, E) and SOD1 females treated with MyoAAV-sKL at the dose of 1.6×10^{13} vg/kg. (C, F). Mean peak MEP amplitudes for the first (G) and second component (H) observed during TA recordings (I, L). MEP/CMAP amplitude ratio for both components (J, K). Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#), WT vs. SOD1 Null (\$) and WT Null vs. WT sKL (&). (n = 14 WT Null, 23 WT sKL, 25 SOD1 Null, 38 SOD1 sKL). Data is shown as mean $\pm$ SEM.

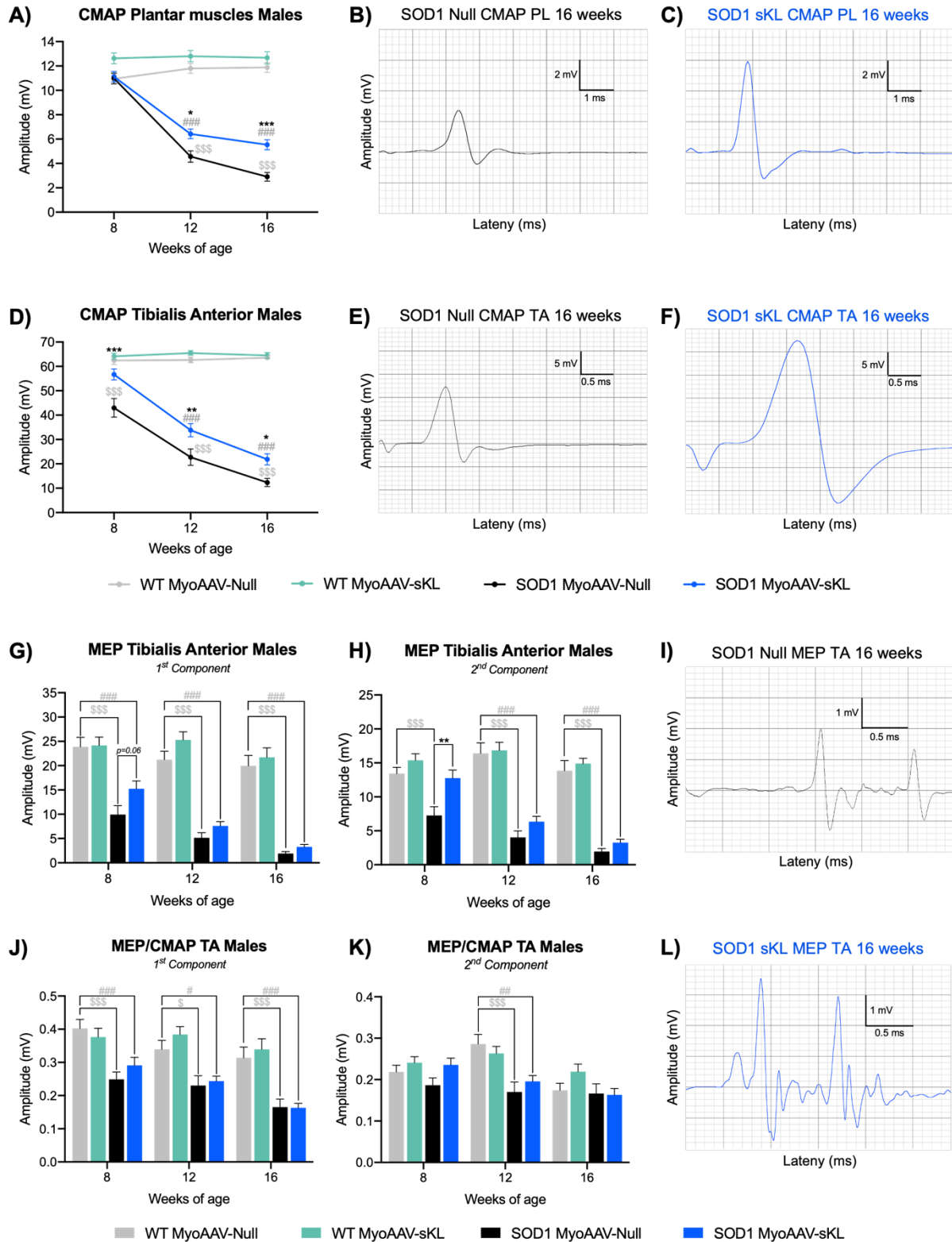


Figure 29. SOD1^{G93A} male mice treated with MyoAAV-Des-sKL show a preserved neuromuscular function. Mean male CMAP amplitudes of the PL (A) and TA muscles (D) with a representative recording for SOD1 Null (B, E) and sKL-treated mice (C, F). MEP amplitudes for the first (G) and second component (H) obtained in TA recordings (I, L). MEP/CMAP amplitude ratio for both components (J, K). Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*), WT Null (#), WT vs. SOD1 Null (\$), and WT Null vs. WT sKL (&). (n = 17 WT Null, 22 WT sKL, 17 SOD1 Null, 27 SOD1 sKL). Data plotted as mean $\pm$ SEM.

In male $SOD1^{G93A}$ mice, the differences in MEP amplitude between sKL- and Null-treated groups only became apparent at the youngest age tested, before the onset of motor symptoms. These disparities persisted, maintaining slightly higher amplitudes throughout the progression of the disease, although they did not reach statistical significance (Fig. 29 G-H and L). In accordance, the MEP/CMAP ratio is only inclined to be notably higher at 8 weeks of age.

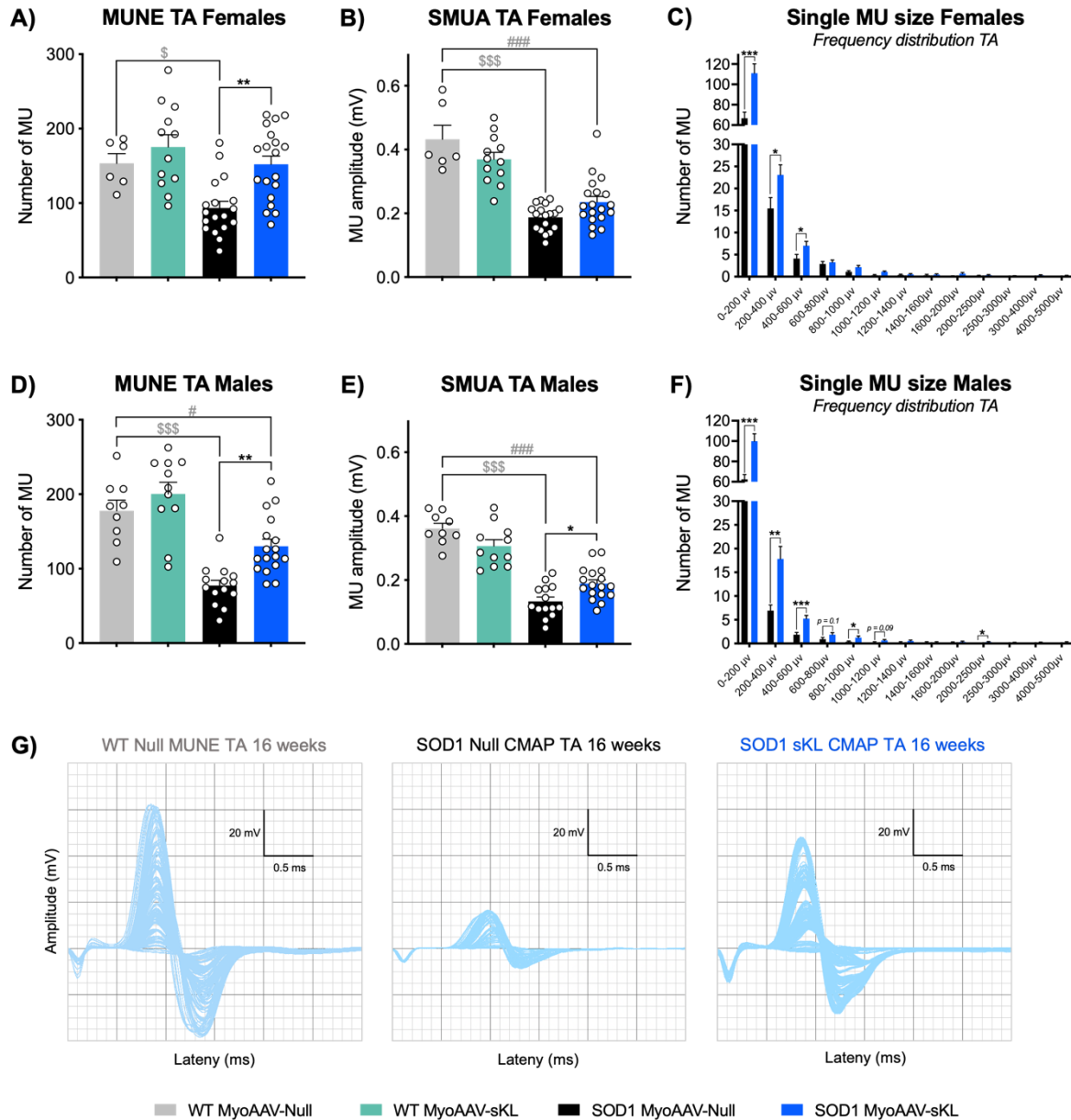


Figure 30. Conservation of neuromuscular function in sKL-treated $SOD1^{G93A}$ mice can be attributed to preservation in the number and size of motor units. Electrophysiological motor unit number estimation (MUNE, A, D) and mean amplitude of single motor units (SMUA) for the TA muscles at 16 weeks (B, E) with representative incremental recordings (G) (Two-way ANOVA with Tukey's post-hoc test). Size distribution analysis of individual motor units in $SOD1^{G93A}$ Null mice compared to treated mice. One-way ANOVA with Tukey's comparisons, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; $SOD1$ sKL relative to $SOD1$ Null (*) or to WT Null (#), and WT relative to $SOD1$ Null (\$); for females $n = 6$ WT Null, 12 WT sKL, 18 $SOD1$ Null, 19 $SOD1$ sKL, and for males $n = 6$ WT Null, 12 WT sKL, 18 $SOD1$ Null, 19 $SOD1$ sKL). Values are shown as mean $\pm$ SEM.

The improvement in neuromuscular function observed in SOD1^{G93A} mice can be explained by a significant preservation in the number of functional motor units (Fig. 30G). This preservation is evident in sKL-treated TA muscles at 16 weeks of age in both females (152.1±11 vs. 93.4±8.9) and males (130.2±9.5 vs. 77.2±7.1) compared to the null-treated mice (Fig. 30A, D). Additionally, the treatment promoted a marginal increase in the amplitude of single motor units in female mice (0.23±0.01 vs. 0.18±0.01 mV), which was statistically significant in SOD1^{G93A} males (0.19±0.01 vs. 0.13±0.01 mV, Fig. 30B, E). These results were corroborated by the size-frequency distribution of the TA motor units, revealing a rightward shift in the treated group (Fig. 30C and F). Overall, the findings suggest that the treatment is maintaining the number of motor units, although they are still experiencing a reduction in size.

3.2.2. Muscle sKL overexpression induced by MyoAAV vectors improves motor performance, delays disease onset, and extends survival

The intravenous administration of MyoAAV vectors and the consequent overexpression of sKL in muscles did not improve the body weight gain of SOD1^{G93A} mice or prevent the decline noted at the late stage (Fig. 31A, B). Nevertheless, in contrast to the decline observed with AAV8-Des-sKL vectors at the high dose, no changes were evident compared to SOD1^{G93A} mock mice.

In treated SOD1^{G93A} animals the time spent on the rotarod test was longer than in untreated animals throughout the disease, demonstrating statistical differences at 16 weeks of age, when most of the untreated animals failed to meet the cutoff time (Fig. 31C, D). The strength of the treated animals was also consistently maintained during disease progression, showing statistical significance from onset to late stages when the force began to differentiate from WT controls (Fig. 31E, F). At 16 weeks of age, the treadmill running test was conducted to assess the maximum speed at which the mice were able to run (Fig. 31G, H). The tests indicated that treated SOD1^{G93A} female mice tended to run faster (23±1.8 cm/s) while treated male mice ran at a significantly higher speed (26.74±1.4 cm/s) compared to matched SOD1^{G93A} null-injected animals (18.5±1.6 and 15.4±2.4 cm/s, respectively).

Importantly, the overexpression of sKL preserved the mass of the gastrocnemius muscle at the end stage (Fig. 31I, J), both in males (112.1.9±5.9 mg) and females (90.9±3.6 mg), compared to SOD1^{G93A} mock-treated mice (85.8±3.7 and 69.1±5 mg, respectively), related to the maintenance of muscle innervation.

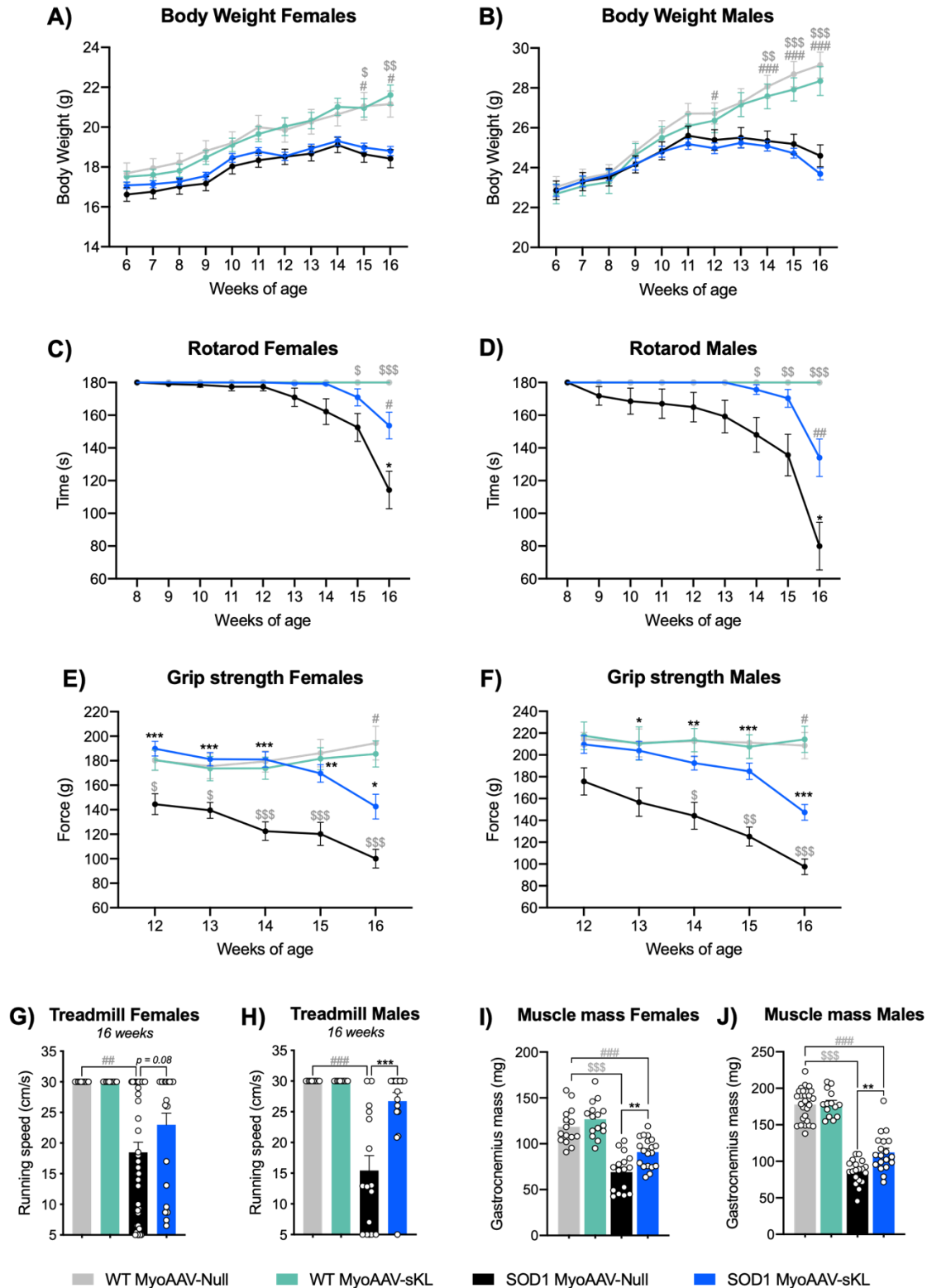


Figure 31. MyoAAV-Des-sKL gene therapy preserves muscle mass and results in broad functional motor improvements in SOD1^{G93A} mice. KL overexpression had no impact on body weight (A, B), but it led to improved performance on the rotarod (C, D), increased running speeds in the treadmill at 16 weeks (G, H), and enhanced grip strength (E, F) in SOD1^{G93A} mice (two-way ANOVA test, n = 10-25 WT Null, 10-26 WT sKL, 15-39 SOD1 Null and 25-50 SOD1 sKL). (I, J) The mass of gastrocnemius muscles was preserved at the end stage (One-way ANOVA test, n = 15-17 WT Null, 14-15 WT sKL, 16-19 SOD1 Null, and 19 SOD1 sKL). Data graphed as mean ± SEM and Tukey's multiple comparisons are as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; SOD1 sKL vs. SOD1 Null (*), or vs. WT Null (#), and WT Null vs. SOD1 Null (\$).

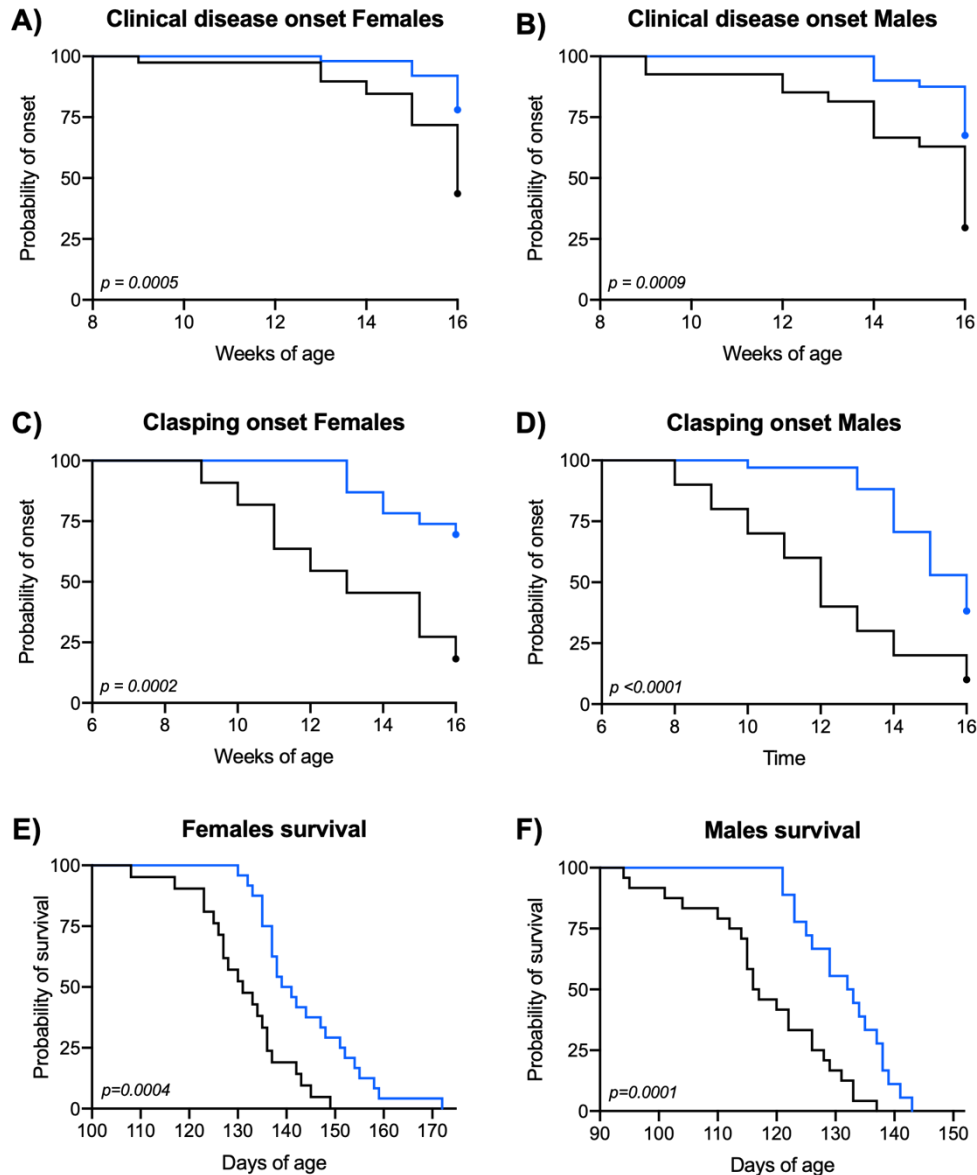


Figure 32. Secretion of sKL from muscles delays disease onset and prolongs survival in $SOD1^{G93A}$ mice. sKL treatment delayed the clinical disease onset determined by rotarod (A, B) (females $n = 39$ SOD1 Null, 50 SOD1 sKL; males $n = 27$ SOD1 Null, 39 SOD1 sKL), and the onset of the clasp phenotype (C, D) (females $n = 22$ SOD1 Null, 23 SOD1 sKL; males $n = 20$ SOD1 Null, 35 SOD1 sKL). Kaplan-Meier median survival (E, F) was significantly extended in SOD1^{G93A}-sKL females ($n = 21$ SOD1 Null, 24 SOD1 sKL) and males ($n = 24$ SOD1 Null, 18 SOD1 sKL) versus control mice. The Log-rank (Mantel-Cox) test was performed for comparisons.

Moreover, these motor improvements in treated animals were reflected in delayed disease onset, as determined by the rotarod test (Fig. 32A, B). At 16 weeks of age, only $22 \pm 6.1\%$ of the females and $32.5 \pm 7.9\%$ of the SOD1^{G93A} males that were treated with MyoAAV-Des-sKL could not maintain the set time on the rotarod, in contrast to $56.4 \pm 8.2\%$ and $70.4 \pm 9.2\%$, respectively, of the SOD1 mock controls. Tremors and clasp of the hindlimbs can be detected earlier and precede dysfunctions in the locomotor and strength tests (Mead et al., 2011). SOD1^{G93A} animals overexpressing sKL exhibited an evident delay in the onset of clasp response compared to Null-injected controls (Fig. 32C, D).

More importantly, the treatment led to a significant extension in the median lifespan of SOD1^{G93A} animals, with a 6.9% (9 days) increase in females and a 13.7% (16 days) increase in males (Fig. 32E, F). While the median survival increase was modest in treated females, some had prolonged lifespans, with the longest-lived mouse reaching 172 days, in contrast to Null-treated mice that survived up to 149 days.

3.2.3. Secreted KL overexpression protects spinal and cortical MNs, preserves NMJ, and reduces glial reactivity

Histological examination of the TA muscles confirmed Klotho overexpression in treated mice, with a 3.3-fold increase in expression for WT sKL and a 4.6-fold increase for SOD1^{G93A} sKL compared to non-treated matched groups (Fig. 33A, B). ELISA analysis of KL levels in the gastrocnemius muscle of SOD1^{G93A} mice further showed an average 2.7-fold increase after the treatment (Fig. 33G).

Although the concentration of KL displayed substantial variability in serum and spinal cord, it still demonstrated a significant 18-fold increase in the serum of treated SOD1^{G93A} mice, indicating its release to the bloodstream from transduced muscles (Fig. 33H, I). Higher sKL levels were detected in the serum of treated SOD1^{G93A} males (93.8±22 pg/mL) than in females (41.32±20.8 pg/mL) and were almost undetectable in SOD1^{G93A} Null mice of both sexes.

On the contrary, in the gastrocnemius muscle, female SOD1^{G93A} mice exhibited higher basal levels (34.5±3.2 pg/mg) than males (20.6±4 pg/mg), even after the treatment (91.3±18.4 and 50.1±10.2 pg/mg, respectively). In the spinal cord, no significant changes were observed in KL concentration, as sKL does not cross the BBB and the desmin promoter should avoid expression in this tissue.

Importantly, the muscle-restricted overexpression of sKL led to a preservation in the number of occupied endplates (78.25±3.5%) at the late stage of the disease (Fig. 33C, D), in comparison to the non-treated group (47±3.9%). The therapy also promoted protection to the MNs (Fig. 33E, F) in the ventral horn of the spinal cord (16.1±0.7, average ± SEM of MNs per section), approximately doubling the counts observed in SOD1^{G93A} mock mice (8.3±0.7). Despite these improvements, no correction was observed in the increased circulating levels of NFL, a biomarker for axonal damage.

No differences in innervation or MN numbers were observed between mock and sKL-treated WT animals. Taken together, these outcomes provide morphological confirmation of the increased CMAP amplitudes and motor unit number detected *in vivo* in the electromyography tests.

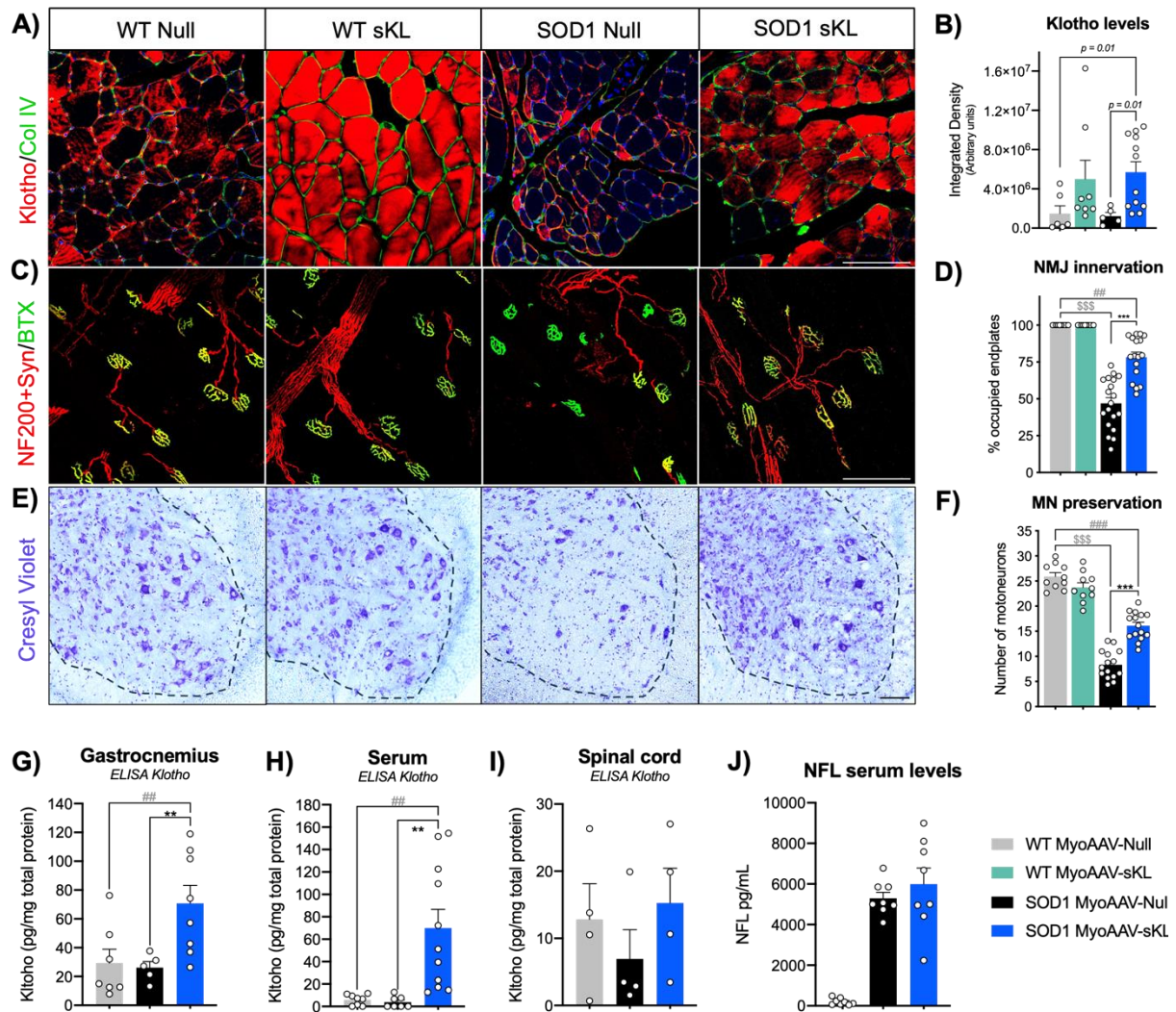


Figure 33. sKL is overexpressed in skeletal muscles, preserving NMJ and protecting spinal MNs in SOD1^{G93A} mice. MyoAAV vectors efficiently overexpress Klotho in the muscles as detected by immunohistochemistry (A, B) and ELISA studies (G). This overexpression also leads to increased serum concentrations (H), while the spinal cord does not exhibit significant changes (I). sKL preserved innervated NMJ at the gastrocnemius muscle (C, D) and provided protection to the cresyl violet-stained MNs in the ventral horn of the spinal cord at the L4-L5 level (E, F), but failed to normalize neurofilament light chain serum levels (J) (One-way ANOVA, Tukey's multiple comparisons are as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#), and WT Null vs. SOD1 Null (\$)). Images scale bar = 100 μm. Mean ± SEM).

Neuroglial reactivity was evaluated in the spinal cord at 16 weeks of age, revealing a reduction in microglial activation (2.86-fold, Fig. 34A, B), in astrocyte reactivity (3.62-fold, Fig. 34C, D) and vimentin expression (2.98-fold, Fig. 34E, F), as previously observed for the AA8-Des-sKL therapy. Secreted KL overexpression in WT mice did not induce significant alterations in the levels of these glial markers. This supports the notion that MyoAAV delivery of sKL in muscles is also able to mitigate the characteristic inflammation hallmarks in the SOD1^{G93A} mouse model.

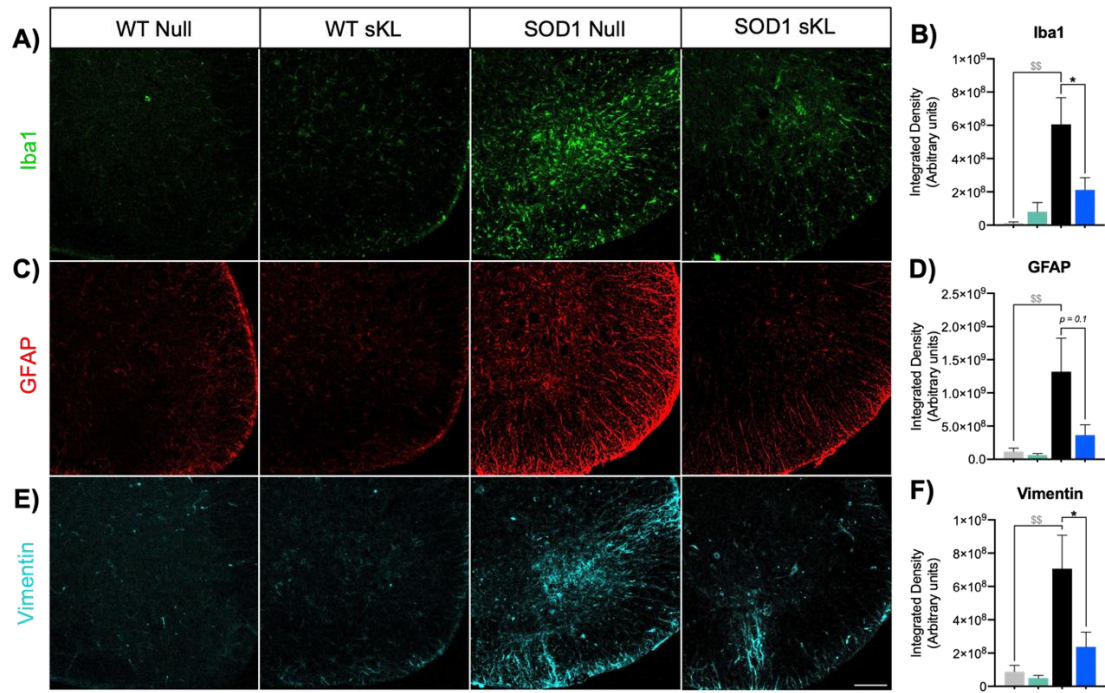


Figure 34. Muscle secretion of sKL protects the spinal cord from neuroglial reactivity in ALS. Representative images of activated microglia labeled against Iba1 (A), reactive astrocytes detected with GFAP (C), and the prototypical inflammation marker vimentin (E). Fluorescence intensity quantification analyses of the inflammatory markers reveal a prominent downregulation in treated SOD1^{G93A} mice. (B, D, F). Two-way ANOVA with Tukey post-hoc test for comparisons; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#), and WT Null vs. SOD1 Null (\$), $n = 5/\text{group}$. Scale bar = 100 μm . Data plotted as mean $\pm$ SEM.

Considering that sKL preserved the amplitude of MEP and spinal MNs, we quantified the number of pyramidal neurons in layer V of the primary motor cortex in female mice. Cortical layer V neurons were less numerous in SOD1^{G93A} Null animals (1211 ± 50.7) compared to WT mice (1441 ± 54.5) and had a smaller cell body, being particularly significant in posterior regions of the cortex (Fig. 35). We found a significant rescue of layer V neurons in sKL-treated SOD1^{G93A} mice (1451 ± 56.6), indicating that the preservation in MEPs is in part due to the preservation of UMNs.

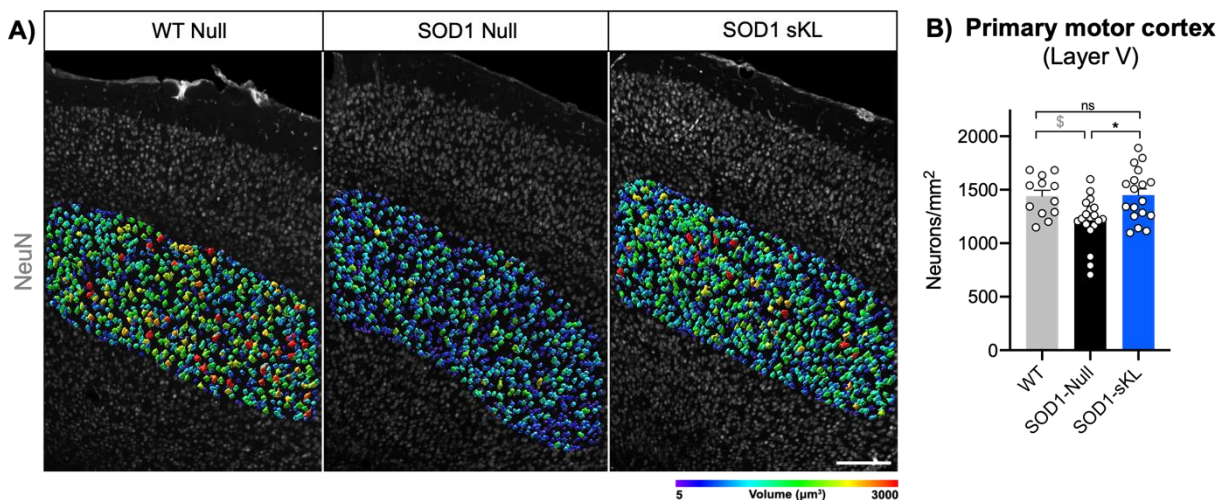


Figure 35. Muscle secretion of sKL protects cortical MNs. (A) Representative NeuN stains of brain coronal sections. The primary motor cortex is shown with neurons in layer V colored according to their volume (scale bar = 100 μ m). (B) Neuronal density in layer V of the primary motor cortex of WT, SOD1^{G93A} Null, and SOD1^{G93A} sKL female mice at 16 weeks. Analysis was performed with Imaris software to quantify neurons and apply a size cut-off. Linear mixed models (LMM) were used for comparisons, incorporating a random effect to accurately accommodate the multiple measurements obtained per mouse; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data is plotted as mean number of neurons per section $\pm$ SEM ($n = 3$ WT, 5 SOD1 Null, and 5 SOD1 sKL, with 4 sections separated at least 200 μ m).

3.3. MyoAAV-mediated muscle overexpression of Klotho at a symptomatic stage

After demonstrating the beneficial effects of sKL in the ALS mice, we considered it clinically relevant to assess the treatment of SOD1^{G93A} mice delivered later, at the symptomatic stage. At the 11th week of age, SOD1^{G93A} mice received tail vein injections containing 1.6×10^{13} vg/kg of the MyoAAV vectors. At this age neuromuscular impairment is already evident, normally manifesting as asymmetrical hindlimb display and claspings, tremors, angle at the ankle joint, diminished strength, and/or an increased susceptibility to fall off the rotarod (Mead et al., 2011). Monitoring of the animals was performed following the protocol outlined in Fig. 36 and, at 16 weeks, samples were collected for histological studies.

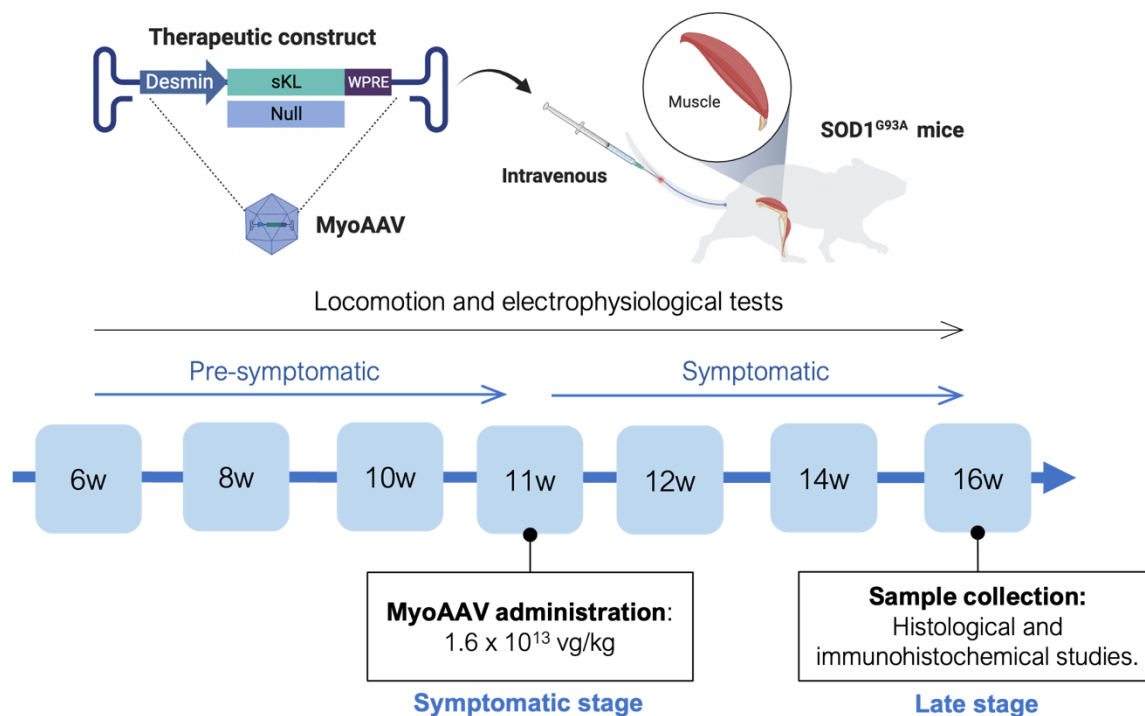


Figure 36. Experimental design for the treatment of symptomatic SOD1^{G93A} mice with MyoAAV-Des-sKL. Animals were administered systemically with MyoAAV vectors at the 11th week of age at a dose of 1.6×10^{13} vg/kg. Mice were monitored weekly by locomotion tests and grip strength, and biweekly by electrophysiological studies. The samples were harvested at 16 weeks of age for histological analyses (females $n = 10$ WT Null, 16 SOD1 Null, 10 SOD1 sKL; males $n = 15$ WT Null, 13 SOD1 Null, 9 SOD1 sKL).

3.3.1. Secreted KL overexpression slows down the decline in neuromuscular function of SOD1^{G93A} mice

Electrophysiological follow-up of SOD1^{G93A} mice treated at clinical disease onset evidenced that the progressive decline in CMAP amplitudes of PL and TA muscles was notably attenuated shortly after the administration of the treatment. In treated female SOD1^{G93A} mice, CMAP amplitude reached significant differences at 16 weeks for both PL (7.2±1 mV) and TA muscles (35.4±5.4 mV), compared to null-injected controls (4.4±0.5 and 19.8±2.2 mV, respectively; Fig. 37A, B). In males, the preservation effect on CMAPs was also evident after administration (Fig. 38A, C), yet at 16 weeks it only remained significant for PL muscles (6.8±1.4 vs. 3.5±0.7 mV).

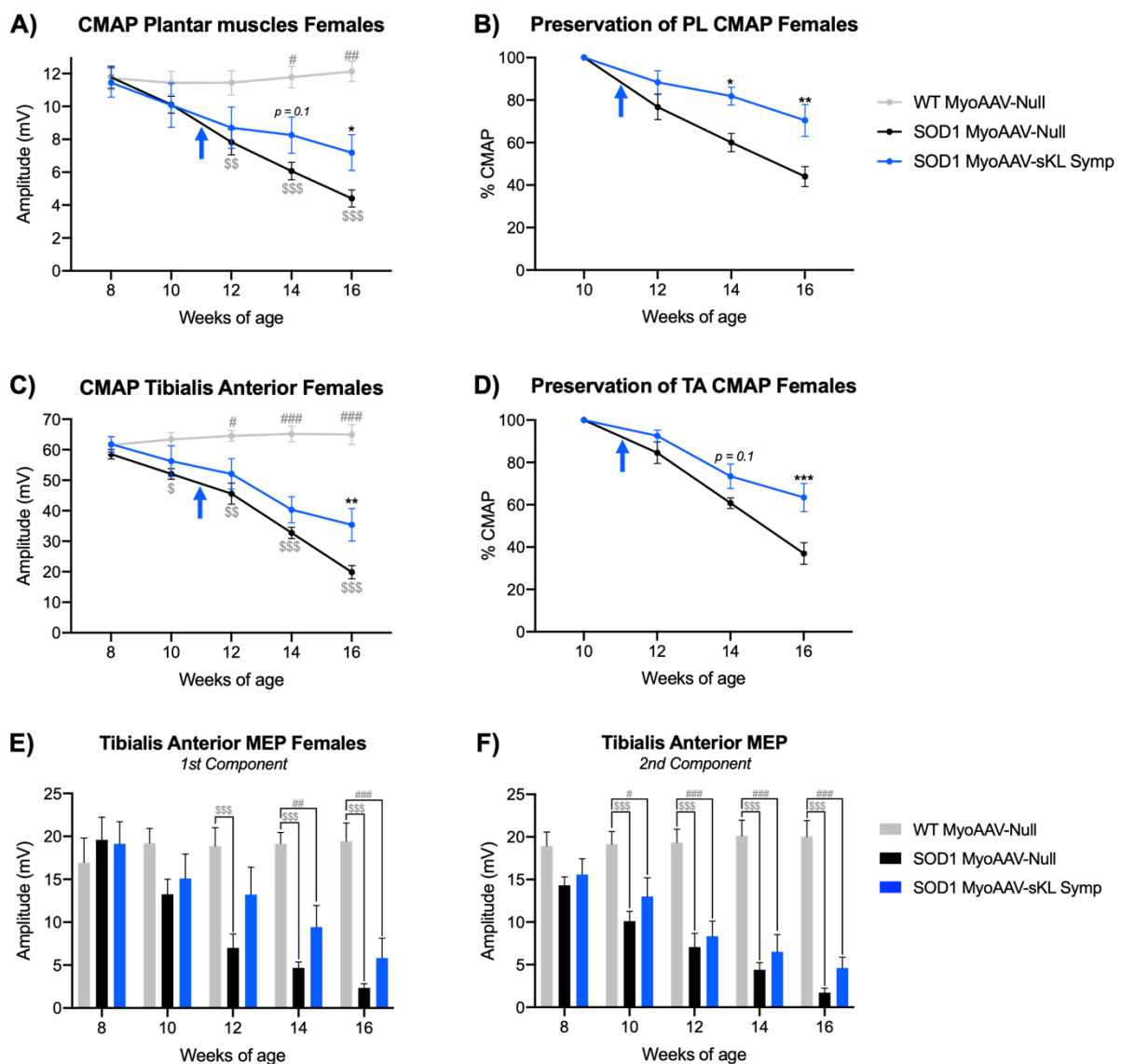


Figure 37. Muscle overexpression of sKL preserves the neuromuscular function in symptomatic SOD1^{G93A} female mice. Absolute CMAP amplitudes for the PL (A) and TA muscles (C). CMAP expressed as the percentage of preservation relative to the week preceding the treatment (C, D). Mean peak MEP amplitudes for the first (E) and second component (F) recorded at the TA muscle. Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#) and WT Null vs. SOD1 Null (\$). (n = 7 WT Null, 11 SOD1 Null, 9 SOD1 sKL). Arrows indicate the time point of administration. Data is shown as mean $\pm$ SEM.

Calculating the CMAP preservation ratio for each animal relative to the week before administration reveals an effective mitigation of CMAP amplitude decline. At the end stage, in females, we achieved a preservation of $70.4 \pm 7.5\%$ for PL muscle and $63.4 \pm 6.6\%$ for TA muscle, in contrast to $44\% \pm 4.7\%$ and $36.9 \pm 5\%$, respectively, in SOD1^{G93A} mock equivalents (Fig. 37B, D).

Similarly, in males, this preservation was $68.3 \pm 11.4\%$ vs. $38.2 \pm 5\%$ for PL muscle and $54.9 \pm 9\%$ vs. $35.7 \pm 3.2\%$ for TA muscle, comparing SOD1^{G93A}-sKL versus SOD1^{G93A}-Null (Fig. 38B, D). Regarding the MEPs, while indicating a trend towards preservation, treatment with sKL was unable to significantly maintain the amplitude of the potentials for both components at this stage in either males or females (Fig. 37E, F and Fig. 38E, F).

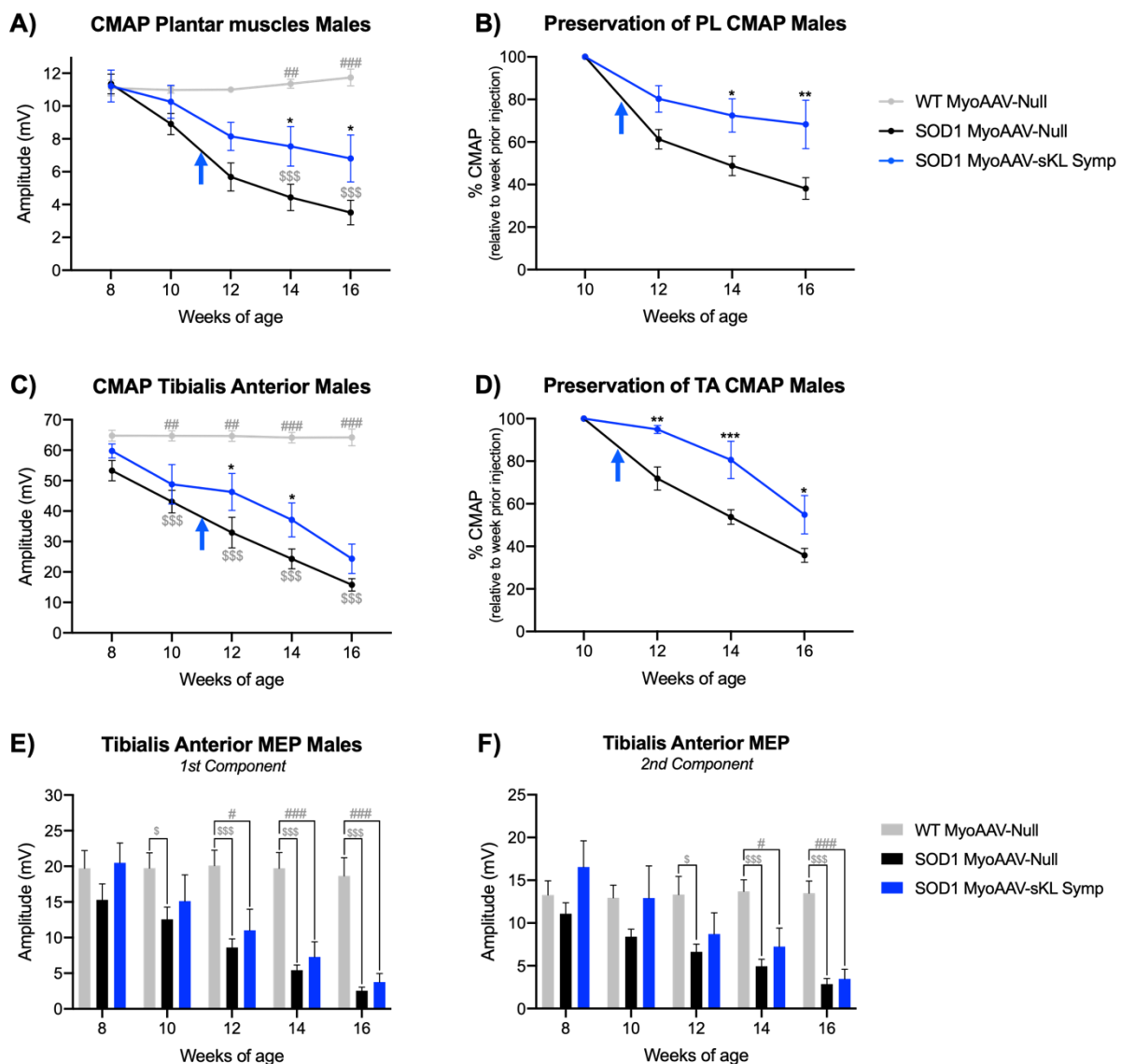


Figure 38. Muscle overexpression of sKL preserves the neuromuscular function in symptomatic SOD1^{G93A} male mice. CMAP amplitude values of the PL (A) and TA muscles (C) and CMAP represented as the percentage of preservation relative to the week before the treatment (C, D). Average maximum MEP amplitudes for the first (E) and second component (F) recorded at the TA muscle. Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#) and WT Null vs. SOD1 Null (\$). (n = 10 WT Null, 11 SOD1 Null, 9 SOD1 sKL). Arrows point to the age of administration. Data presented as mean $\pm$ SEM.

3.3.2. Secreted KL muscle overexpression protects MNs and preserves NMJ while sustaining motor function in symptomatic SOD1^{G93A} mice

Similar to the earlier findings, the outcomes observed in nerve conduction studies are related to a protective effect on the number of surviving spinal MNs in SOD1^{G93A} mice treated at the symptomatic stage (12.71 ± 0.7 , mean $\pm$ SEM number of MNs per slice). This stands in contrast to mock-treated controls (8.33 ± 0.7) when analyzed at 16 weeks of age (Fig. 39A, B).

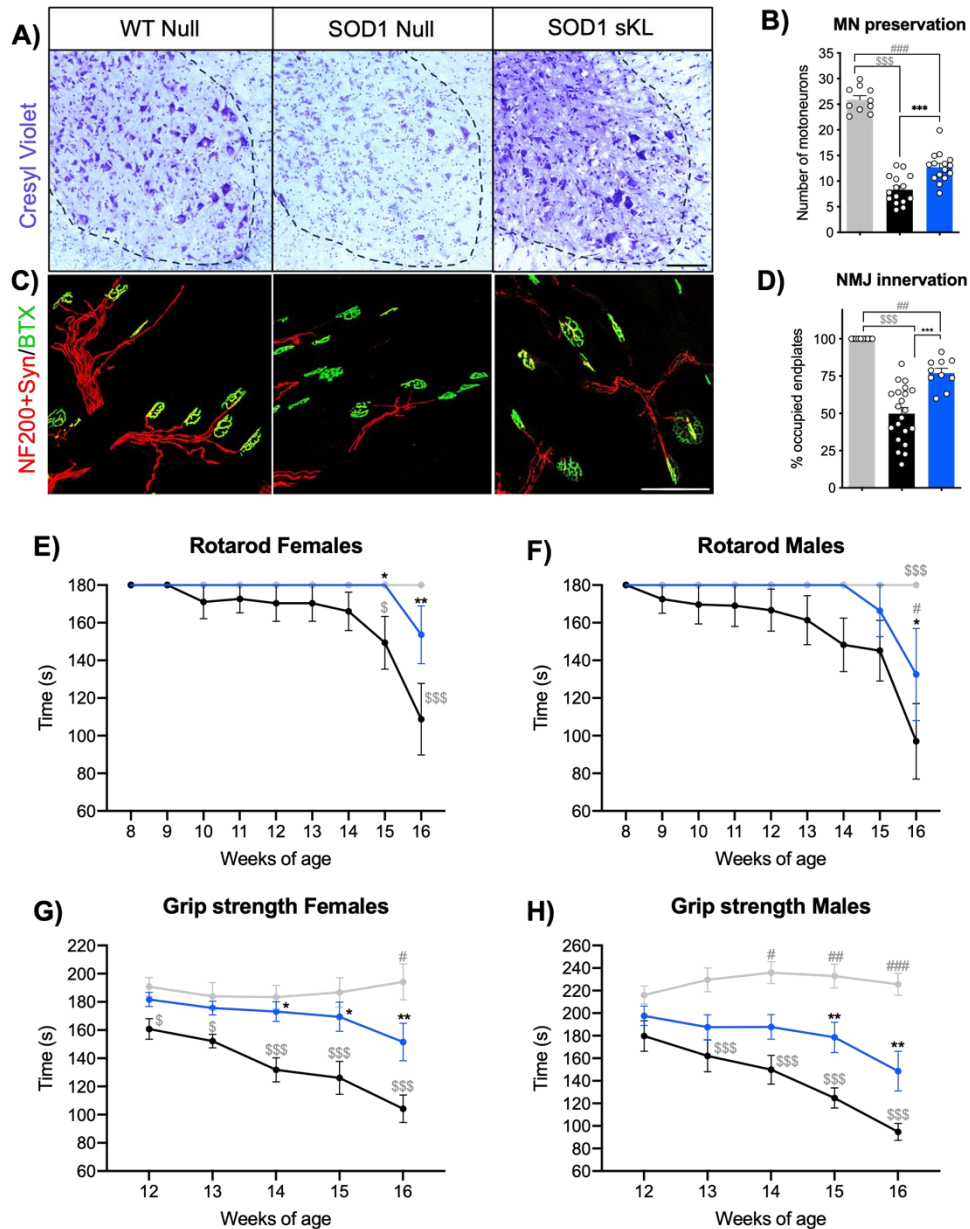


Figure 39. sKL overexpressed in the muscles of symptomatic SOD1^{G93A} mice preserves NMJ and protects spinal MNs resulting in motor improvements. The late administration of MyoAAV-Des-sKL vectors protects cresyl violet-stained spinal MNs at the L4-L5 level (A, B) and sustains the percentage of innervated NMJ at the gastrocnemius muscle (C, D) ($n = 10$ WT Null, 15 SOD1 Null, 15 SOD1 sKL). This translates to an enhanced motor performance in the rotarod test (E, F) and a preservation in the strength of treated-SOD1 animals (G, H) (females $n = 10$ WT Null, 16 SOD1 Null, 10 SOD1 sKL; males $n = 15$ WT Null, 13 SOD1 Null, 8 SOD1 sKL). Two-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Tukey post-hoc comparisons are: SOD1 sKL vs. SOD1 Null (*) or vs. WT Null (#) and WT Null vs. SOD1 Null (\$). Scale bar = 100 μ m. Mean $\pm$ SEM.

Additionally, there was a preservation in the percentage of innervated NMJ in the treated group ($77 \pm 3.1\%$) versus the untreated SOD1^{G93A} group ($49.9 \pm 4.3\%$) (Fig. 39C, D). We found enhanced performance in the rotarod test (Fig. 39E, F) and a significant preservation of strength at later stages of the disease (Fig. 39G, H). Taken together, these findings demonstrate that even if given during the overt progression of the disease, the overexpression of sKL in muscles has the capacity to reduce the advancement of MN degeneration and promote functional improvements in the SOD1^{G93A} mouse model.

3.4. Widespread overexpression of sKL fails to preserve neuromuscular function in SOD1^{G93A} mice

Considering the increased sKL serum concentration observed in treated SOD1^{G93A} mice (see Fig. 33H), we questioned whether the beneficial effects originated from elevated circulating sKL levels rather than a robust expression in skeletal muscles. To elucidate if the overexpression of sKL was specifically needed in the muscles we conducted a proof-of-concept study in which we intravenously administered a small batch of SOD1^{G93A} female mice with 1.6×10^{13} vg/kg of AAV9-CAG-sKL vectors (Fig. 40). Our aim was to target a widespread, and mostly, liver sKL overexpression and to achieve a stronger secretion to the bloodstream.

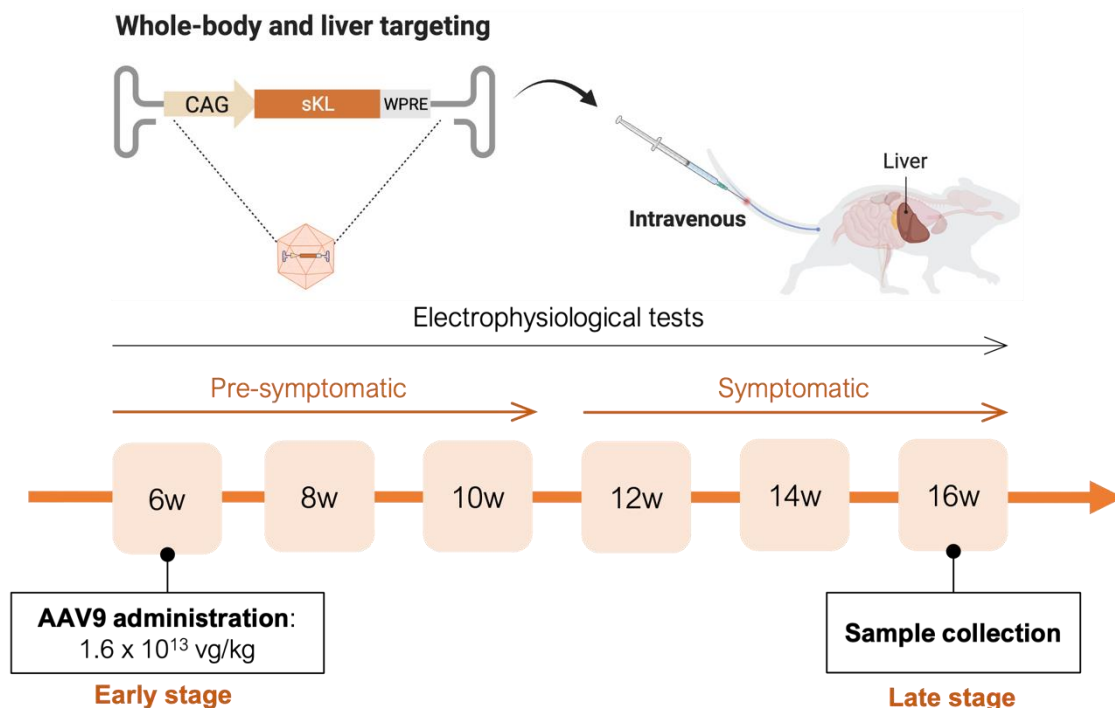


Figure 40. Study design for the systemic administration of AAV9-CAG-sKL in SOD1^{G93A} mice. Animals were administered intravenously at 6 weeks of age at 1.6×10^{13} vg/kg. Therapeutic efficacy was assessed by electrophysiological tests at 8, 12, and 16 weeks of age. (n = 9 WT Null, 12 SOD1 Null, 6 SOD1 AAV9-sKL).

The use of AAV9 vectors and the ubiquitous CAG promoter for whole-body overexpression of sKL resulted in higher KL serum levels compared to the MyoAAV-based approach, showing a 2.5-fold increase (Fig. 41E). However, the similar levels of sKL in the gastrocnemius muscle compared to those of control animals, evidenced the inefficiency of overexpressing sKL in muscles using this strategy (Fig. 41F). This approach failed to preserve the CMAP amplitude of the PL and TA muscles (Fig. 41A, B) and the peak amplitude of the MEPs (Fig. 41C, D) in AAV9-treated $SOD1^{G93A}$ females when compared to the mock group. These data reinforce the need to precisely direct sKL overexpression to the skeletal muscles and highlight the importance of using AAV serotypes with enhanced muscle tropism for this therapeutic strategy.

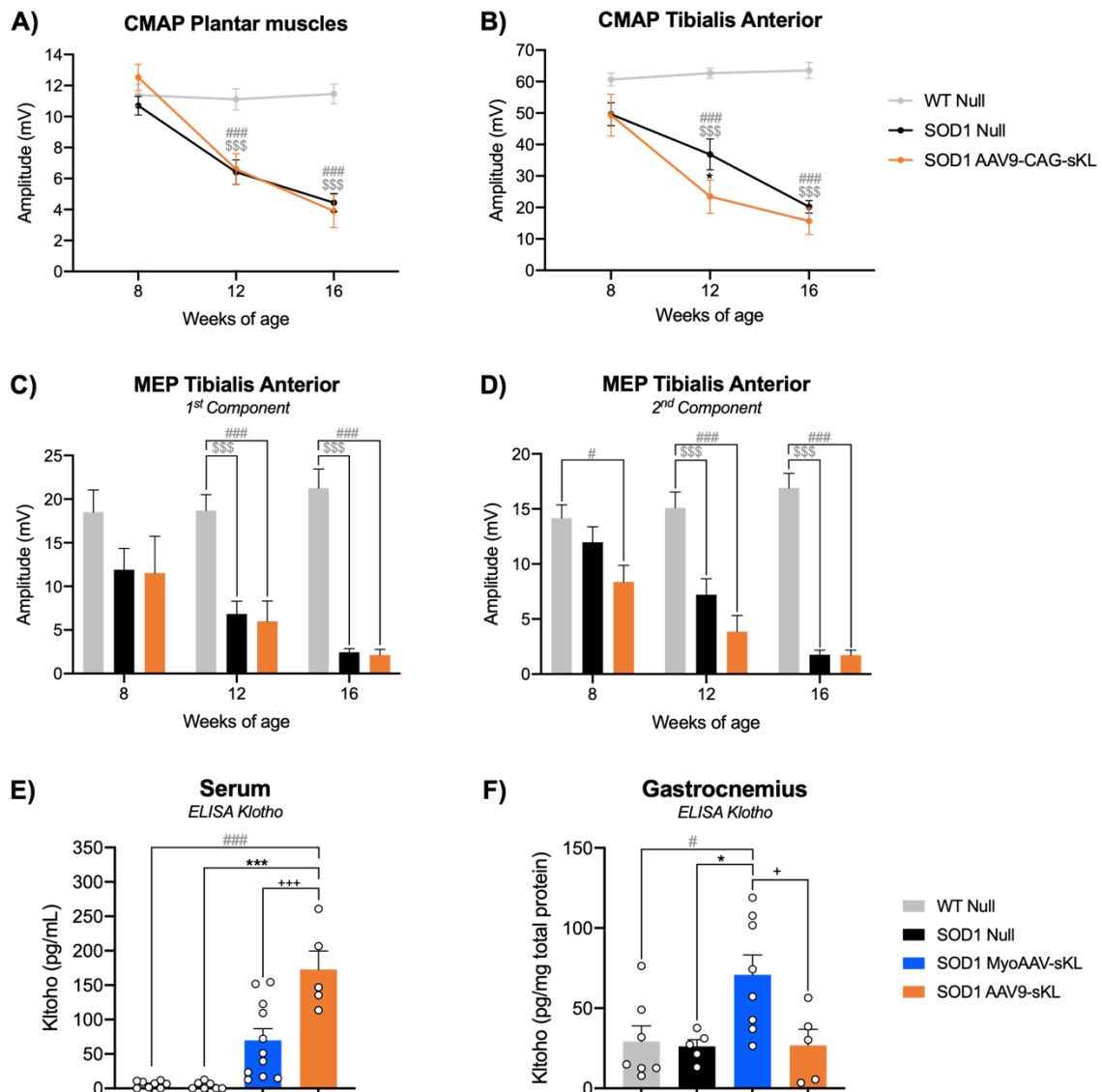


Figure 41. High circulating sKL levels do not preserve neuromuscular function in $SOD1^{G93A}$ female mice. CMAP amplitudes for the PL (A) and TA muscles (B) and mean peak MEP amplitudes for the first (C) and second component (D) recorded at the TA muscle comparing AAV9-CAG-sKL-injected $SOD1^{G93A}$ mice with controls (Two-way ANOVA with Tukey post-hoc test, $n = 9$ WT Null, 12 SOD1 Null, 6 SOD1 AAV9-sKL). Serum (E) and gastrocnemius muscle (F) sKL concentration in Null-, MyoAAV- and AAV9-treated animals. (One-way ANOVA, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$; Tukey multiple comparisons are SOD1 sKL vs. SOD1 Null^{*} or vs. WT Null[#] and WT Null vs. SOD1 Null^{\$} and SOD1 AAV9-sKL vs. SOD1 MyoAAV-sKL⁺. Data is shown as mean $\pm$ SEM.

Chapter IV:

Transcriptomic and proteomic studies unravel
Klotho mechanisms of protection in ALS

4.1. Overexpression of sKL in muscles alters gene expression profile in the spinal cord

4.1.1. Transcriptomic profiling of the spinal cord in sKL- and Null-treated mice

To determine the global effect of muscle secretion of sKL in the transcriptional footprint of the spinal cord, we performed bulk RNA sequencing (RNAseq) studies. We aimed to uncover the mechanisms underlying the neuroprotective effects of sKL and the maintenance of neuromuscular function. Lumbar spinal cords of female mice, including null and sKL WT and SOD1^{G93A} groups treated with MyoAAV vectors at 6 weeks of age, were examined at 16 weeks of age.

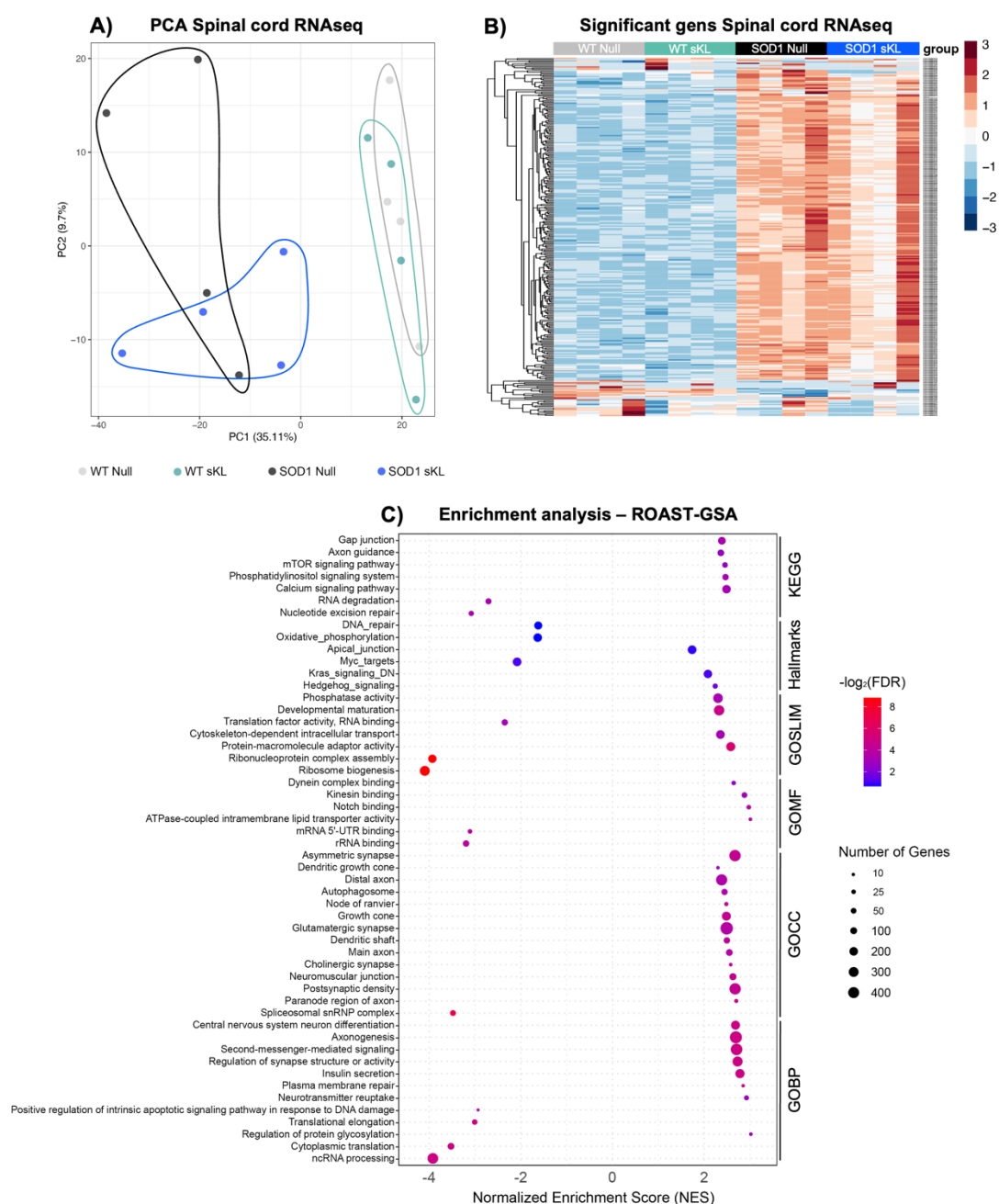


Figure 42. Transcriptomic profiling of the spinal cord of WT and SOD1^{G93A} female mice treated with MyoAAV-Null or sKL vectors. (A) Principal component analysis (PCA) of RNAseq data showing overall gene expression patterns. (B) Heatmap showing differentially expressed genes between the four groups, each column represents a different animal (n = 4). Genes differentially expressed in any group are displayed with their Z-score across all groups. (C) Dot plot of the functional enrichment analysis with the most significant differences in KEGG, Hallmarks, and GO terms comparing SOD1 sKL to SOD1 Null animals. The plot represents major over-represented gene sets at the top and bottom of the transcriptome data.

Bioinformatic analysis showed that these samples grouped in two distinct populations based on the principal components of variance, with WT and SOD1^{G93A} mice clustering together regardless of the treatment (Fig. 42A). This indicates that there are no general characteristic expression profiles between the treated groups in the spinal cord. Differential expression analysis revealed only 5 differentially expressed genes (DEGs) between WT sKL and Null, and 5 DEGs in SOD1^{G93A} sKL mice compared to SOD1^{G93A} null controls ($\log_2$ fold change $>|1|$, FDR <0.1). In contrast, 663 genes were upregulated and 48 were downregulated in SOD1^{G93A} in comparison to WT control mice (Table 3).

In addition, 413 genes were statistically significant and transcriptionally active in SOD1^{G93A} sKL mice versus SOD1^{G93A} Null mice but were not differentially regulated more than 2-fold. sKL mRNA levels were not significantly increased in either SOD1^{G93A} or WT animals with sKL treatment compared to Null treatment, suggesting that no expression of the vectors was achieved in the spinal cord. As observed in both the PCA plot and the heat map of significant genes across the four groups (Fig. 42B), the transcriptional profile of two of the treated SOD1^{G93A} animals appeared to be partially corrected.

Table 3. Differentially expressed genes in the spinal cord of sKL- and Null-treated WT and SOD1^{G93A} mice for the multiple contrasts. ($p_{adj} < 0.1$; |Shrunken FC| > 2).

Contrast	Downregulated DEG	Upregulated DEG
SOD1 sKL_SOD1 Null	12	15
SOD1 Null_WT Null	39	424
SOD1 sKL_WT sKL	30	421
WT sKL_WT Null	33	18
Inter (4 groups)	14	10

We then employed hybrid methods combining ROAST (Rotation Gene Set Testing) with Gene Set Enrichment Analysis (GSEA) to assess whether the significant genes in the spinal cord of treated and non-treated SOD1^{G93A} mice could collectively contribute to differentially activated pathways. Functional annotation showed that genes grouped into 193 Gene Ontology (GO) categories and 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms were significantly differently regulated.

The most differentially regulated and representative GO, Hallmark, and KEGG categories applicable to sKL treatment and ALS biology were plotted with their respective P-values, number of genes measured, and normalized enrichment score (NES) (Fig. 42C).

GSEA revealed a remarkable down-regulation in KEGG and GO categories associated with **RNA degradation, ribonucleoprotein complex assembly, and rRNA and mRNA binding** (Fig. 43A, B, C). These categories primarily included genes related to ribosomal function, those involved in the spliceosome complex, and those encoding RNA processing or interacting proteins. Disturbances in RNA expression, metabolism, and RNA-binding proteins are pathologic hallmarks of ALS, and these effects appear to be reversed at the mRNA level by our treatment.

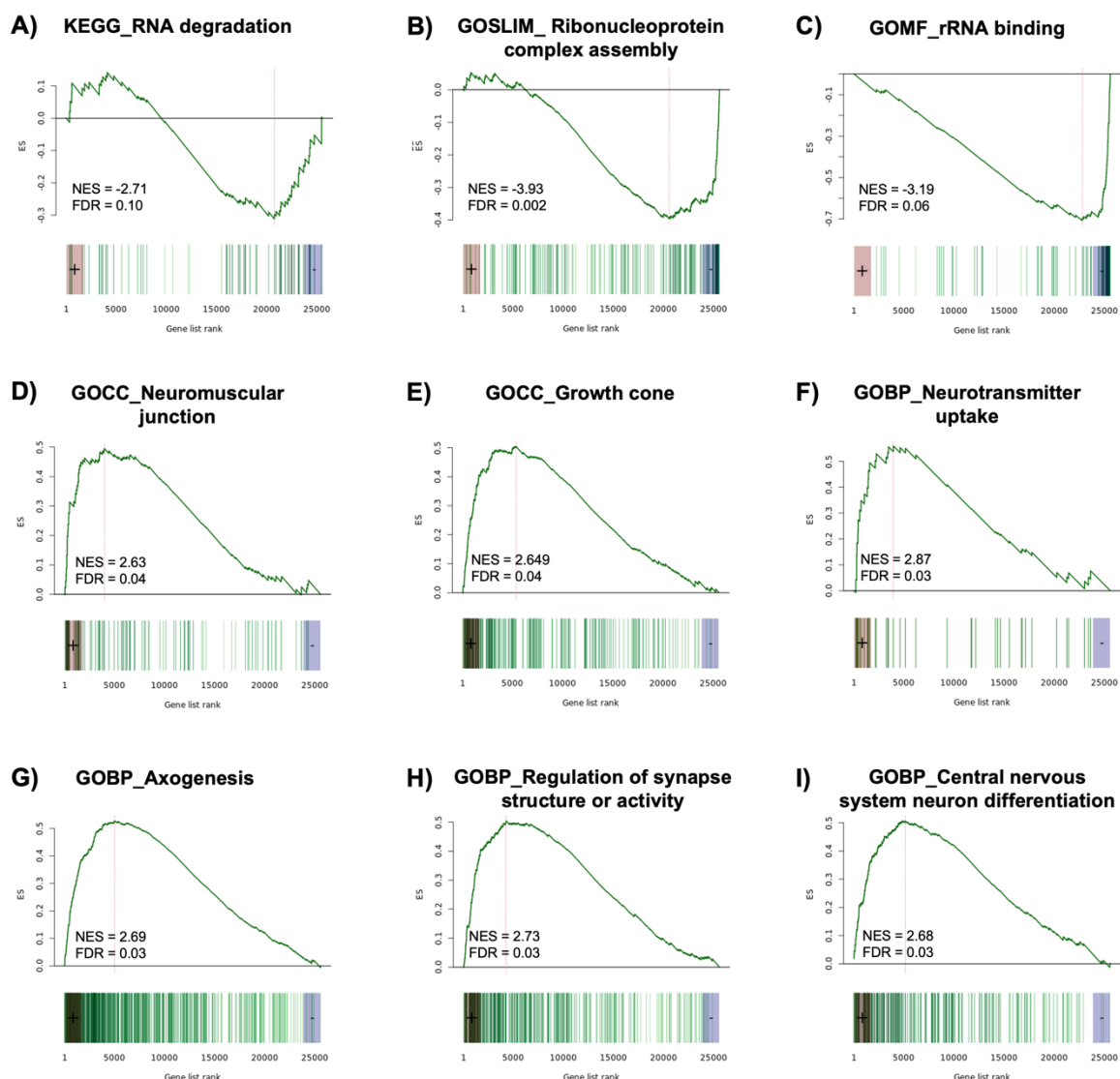


Figure 43. Gene set enrichment analysis (GSEA) depicting linkages of genes and biological concepts in the spinal cord. Downregulated and upregulated GSEA of representative KEGG (A), GOSLIM (B), GOMF (C), GOCC (D, E) and GOBP (F, G, H, I) terms with biological interest in *SOD1^{G93A}* female mice when overexpressing sKL in muscles compared to mock controls. The peak in the plots reflects the enrichment score, indicating whether the gene set is over- (positive) or under-expressed (negative) relative to the entire ranked gene list.

KEGG, GOCC, and GOBP subontologies associated with **axon regeneration**, such as axogenesis, axon guidance, dendritic growth cone, dendritic shaft, and **CNS neuron differentiation** were upregulated in sKL-treated SOD1^{G93A} mice compared with mock counterparts (Figs. 41C and 42E, G, H, I).

These categories shared numerous genes associated with axon guidance and outgrowth, including several semaphorins and netrins, EphA receptor tyrosine kinases, RET receptor, chondroitinase, chondrolectin, ventral anterior homeobox 1 (*Vax1*), and the fasciculation and elongation protein zeta-1 (*Fez-1*). In addition, we found an enrichment in the mRNA of adhesion molecules (*Ncam1*, *Nectin1*, *Chl1*) and cytoskeletal proteins (*Septin7*, *Tubb3*, *Myot*). Oligodendrocyte proteins (e.g., *Olig2* and *Mag*) were also upregulated.

GO annotations associated with **axonal transport** and **cargo-binding proteins**, such as cytoskeleton-dependent intracellular transport, dynein complex binding, or kinesin binding, exhibited a positive NES score (Fig. 42C).

Genes involved in the **regulation of synaptic activity and assembly**, and in **neurotransmitter reuptake** were also positively enriched in the treated animals (Fig. 43F, H). Notable genes in this context include α -synuclein, synaptotagmins 2, 6, 7, and 17, syntaxin binding protein 1, *Unc13a*, *Unc13c*, N-acetyltransferase 8 like, nitric oxide synthase 1 (*Nos-1*), and several genes from the solute carrier family (*Slc22a23*, *Slc29a1*, *Slc30a3*, *Slc4a10*, *Slc6a11*, *Slc6a7*, *Slc24a2*, *Slc25a29* and *Slc12a5*). It is worth noting the tendencies for downregulation of the gene encoding AMPA receptor subunit 1 (*Gria1*) and for the upregulation of the different ADAR family genes in SOD1^{G93A} sKL mice compared to untreated controls.

More importantly, relevant genes related to **neuromuscular junctions** were overrepresented in the spinal cord of mice overexpressing sKL (Fig. 43D), including *Nrg1*, *Erbp2* interacting protein, caveolin 3, *VACht* (*Slc18a3*) and other choline transporters. Messenger RNA expression of light and medium neurofilament polypeptides was decreased in SOD1^{G93A} mice but was restored to WT levels in treated animals.

4.1.2. Protein expression patterns in the spinal cord of sKL and Null-treated mice

To obtain complementary information, proteomic analyses were performed on the same samples, as one hemisection of the lumbar spinal cord was used for RNAseq and the other for protein extraction.

Proteomic studies directly measure proteins present in the samples and thus provide insights into the actual functional entities within the cells, which may not always correlate with mRNA abundance. However, while RNAseq can provide a more comprehensive overview of the entire transcriptome, and cover a wide range of RNA species, proteomics cannot capture the complete proteome.

A total of 9,858 proteins were initially identified by mass spectrometry. After data processing, this set was refined to 6,142 protein groups recognized by more than one unique peptide, and these were subjected to differential abundance analysis. The four groups were contrasted with each other to detect proteins significantly over- or under-represented in the spinal cord (Table 4).

Table 4. Numbers of neutral proteins and proteins significantly over- or underabundant in the spinal cord in each contrast ($p_{adj} < 0.05$; neutral = $-1.5 < FC < 1.5$, up = $FC > 1.5$, down = $FC < 1.5$).

Contrast	Down	Neutral	Up
SOD1 sKL_SOD1 Null	0	6103	0
SOD1 Null_WT Null	33	5742	182
SOD1 Null_WT sKL	13	5834	125
SOD1 sKL_WT Null	2	5846	82
SOD1 sKL_WT sKL	1	5885	60
WT sKL_WT Null	0	5217	0

The hierarchical clustering heatmap of significant proteins ($|FC| > 1.5$ and $p_{adj} < 0.05$) in any of the six contrasts shows two main protein clusters. The first with particularly abundant proteins in the SOD1^{G93A} groups and the second with highly abundant proteins in the WT groups (Fig. 44C). Analogous to the RNAseq results, the PCA and heatmap reveal that two of the SOD1^{G93A} sKL-treated animals have a proteomic profile more similar to that of the WT groups (Fig. 44A, C).

Although no significant differentially expressed proteins were found between Null and sKL SOD1^{G93A} mice with the established thresholds ($|FC| > 1.5$ and $p_{adj} < 0.05$), a correction in the number of up- and downregulated proteins compared to WT mock animals is evident (Table 4 and Fig. 44B). SOD1^{G93A} Null mice had 33 overabundant and 182 underabundant proteins compared to WT Null mice, which is reduced to 2 and 82, respectively, in treated SOD1^{G93A} mice. Additionally, even fewer differentially expressed proteins were observed when comparing sKL-treated WT and SOD1^{G93A} mice.

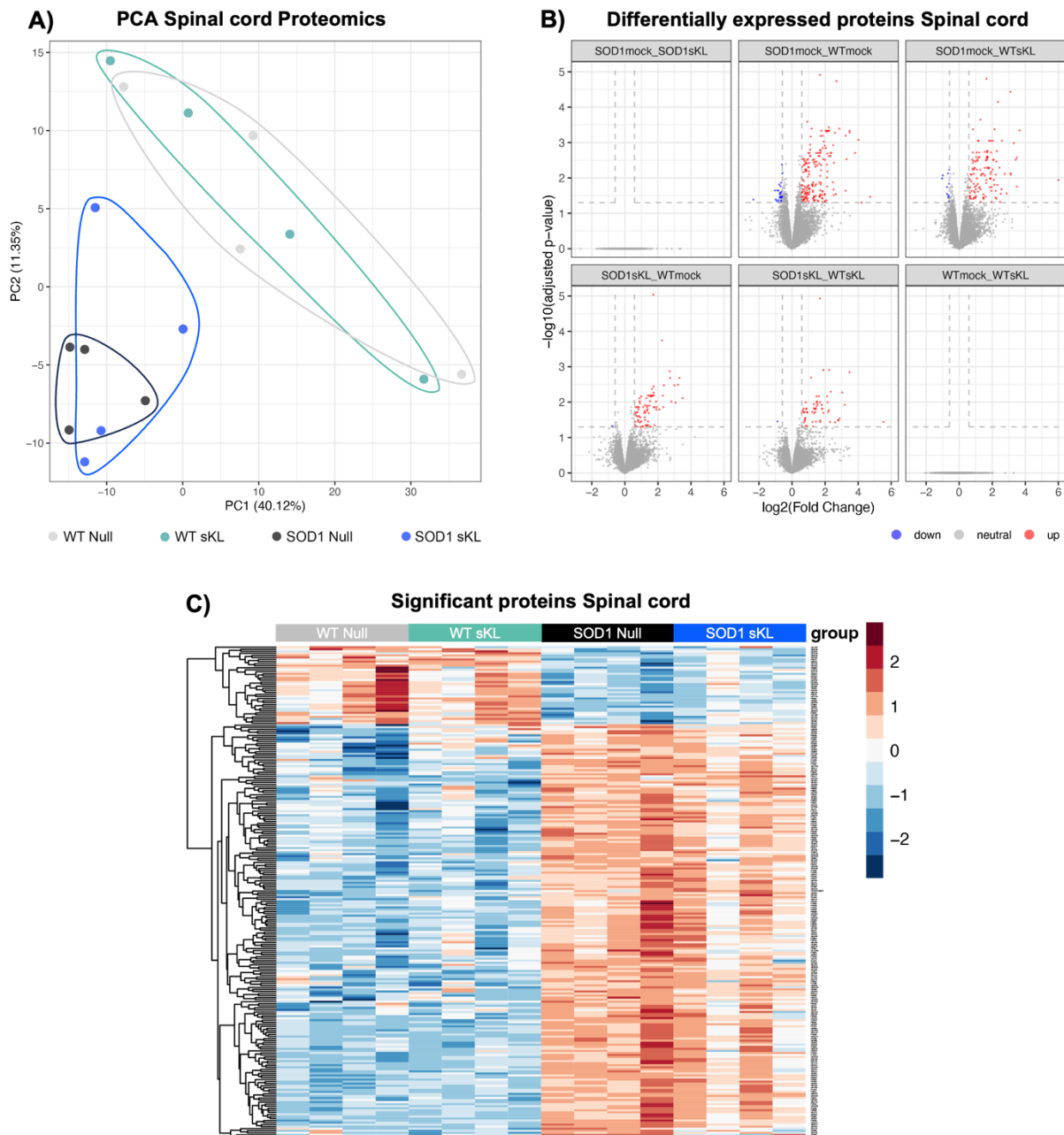


Figure 44. Proteomic analysis of the spinal cord of WT and SOD1^{G93A} female mice treated with MyoAAV-Null or sKL vectors. (A) Principal component analysis (PCA) of proteomic data showing the overall variation of protein abundance in the four biological groups. (B) Volcano plot of differentially expressed proteins when comparing the different groups. The dashed lines represent the significance cutoff. (C) Clustering heatmap of significant proteins for the studied contrasts. Each protein is represented by a row of colored tiles (red, up-regulated; blue, down-regulated), and each animal by a column in the same order as in RNAseq data. ($|FC| > 1.5$ and $p_{adj} < 0.05$, $n = 4/\text{group}$).

We then selected the top 20 proteins with biological significance that were either downregulated or upregulated in the spinal cord of SOD1^{G93A} Null mice and that were partially corrected, corrected, or overcorrected by the treatment (Fig. 45A, B). These dysregulated proteins represent mechanisms that undergo significant alterations in ALS.

Proteins such as α -synuclein, the inward rectifier potassium channel KCNJ16, unc-79 homolog, the acetylcholine autoreceptor CHRM2, cholinesterase, Sema4C, and reticulon-2 are integral to **neurotransmission, excitability, synaptic plasticity, and axonal guidance**. Similarly, chromogranin-A and neuroendocrine convertase-2 play a role in **neuroendocrine function** and the **processing of peptide hormones**.

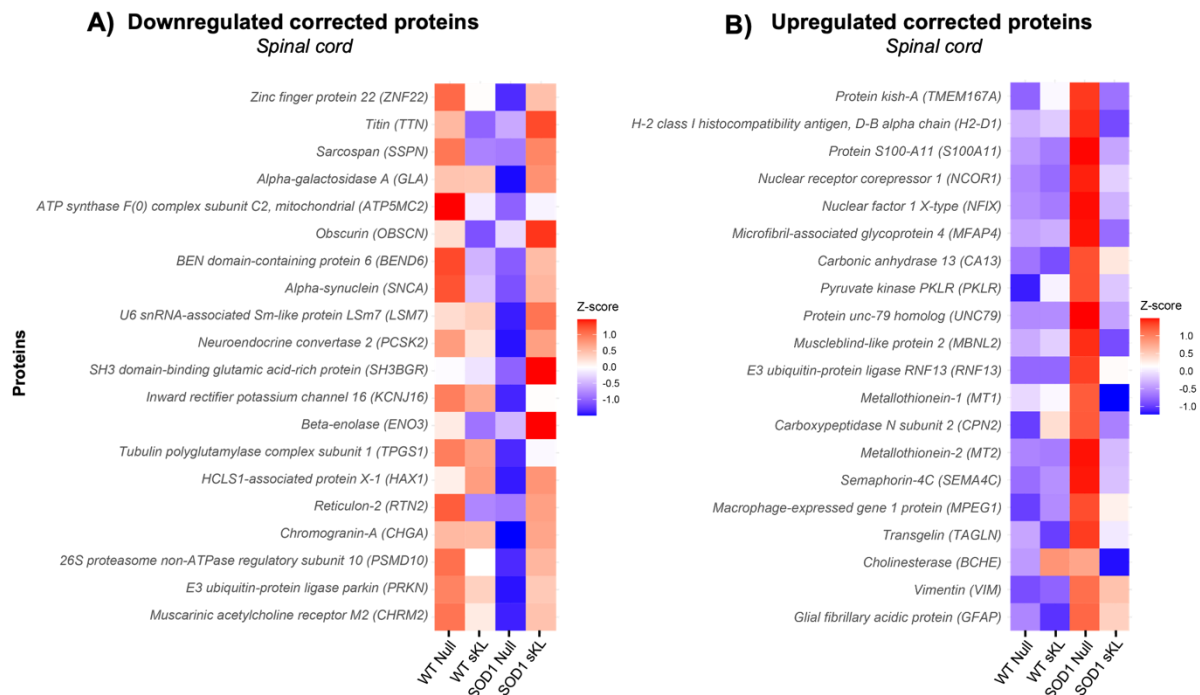


Figure 45. Representative corrected proteins in the spinal cord of sKL-treated SOD1^{G93A} female mice. Top downregulated (A) and upregulated (B) biologically relevant proteins in the lumbar spinal cord of SOD1^{G93A} mice corrected by the treatment.

In addition, proteins associated with **mitochondrial function, mitophagy, or metabolic processes** — including ATP synthase, β -enolase, parkin, HAX-1, carbonic anhydrase, and pyruvate kinase PKLR — were found to be dysregulated in SOD1^{G93A} mice but were corrected by muscle overexpression of sKL.

This analysis highlights several proteins associated with **RNA perturbations** (SM-like protein LSM7, muscleblind-like protein 2, HAX-1) and **proteostasis** (adaptor SH3BGR, gankyrin, NCOR1, NFIX, RNF13 ubiquitin-protein ligase, carboxypeptidase N) that exhibit differential regulation between treated and untreated mice. Specifically, several **neuroimmune system-related proteins**, including H-2 class I histocompatibility antigen (H2-D1), S100A molecules, macrophage-expressed gene 1 protein (MPEG1), monocyte chemoattractant protein-1 (MCP1), FGFr, α_2 -macroglobulin, and interferon-induced IFIT-family protein. Moreover, vimentin and glial fibrillary acidic protein (GFAP) were downregulated by the treatment, as we previously demonstrated by immunohistochemistry in the histologic analyses (Fig. 34A-D).

Additionally, proteins related to the **cytoskeleton** and **extracellular matrix** (tubulin-polyglutamylase complex, microfibril-associated glycoprotein 4, and transgelin) and those associated with **oxidative and metal-induced toxicities** (protein Kish-A and metallothioneins) were also corrected. Surprisingly, titin, sarcospan, and obscurin, proteins traditionally associated with muscle contraction, muscle membrane maintenance, and sarcomere organization, were identified in the spinal cord, and showed some of the most positive fold-change between SOD1^{G93A} mock and sKL-treated mice.

4.2. Overexpression of sKL in the muscles results in rectified gene expression patterns

4.2.1. Transcriptomic profile of the gastrocnemius muscle in sKL- and Null-treated mice

Having shown that increased levels of sKL in muscles lead to functional improvements, we performed RNAseq analysis in muscles to investigate the gene expression signature associated with the therapeutic effects. Gastrocnemius muscle samples were collected at 16 weeks of age from the same animals in which omics studies were performed in the spinal cord.

In this case, PCA identified three distinct groupings within the RNAseq data. These groupings include the SOD1^{G93A} null group, the WT group irrespective of administration, and an intermediate cluster represented by SOD1^{G93A} sKL animals that lies between the two previous clusters (Fig. 46A). This is also illustrated in the heatmap depicting the significant genes between the four groups, with SOD1^{G93A} sKL animals showing color hues more similar to those of WT mice (Fig. 46B).

Table 5. Differentially expressed genes in the gastrocnemius muscle of sKL- and Null-treated WT and SOD1^{G93A} mice for the different contrasts. ($p_{adj} < 0.1$; |Shrunken FC| > 2).

Contrast	Downregulated DEG	Upregulated DEG
SOD1 sKL_SOD1 Null	82	37
SOD1 Null_WT Null	272	449
SOD1 sKL_WT sKL	31	157
WT sKL_WT Null	20	24
Inter (4 groups)	17	23

In WT mice, differential expression analysis uncovered 45 DEGs upon the overexpression of sKL in the muscle (log2 fold change >|1.5|, FDR < 0.1). A total of 746 DEGs were found in SOD1^{G93A} mice compared to WT mice. In contrast, only 123 DEGs were detected in SOD1^{G93A} sKL versus Null mice, consisting of 45 upregulated and 78 downregulated genes (Table 5).

An additional 157 DEGs were identified as statistically significant between SOD1^{G93A} sKL and Null mice, though this alteration exhibited less than a 2-fold change. Of these DEGs, sKL exhibited a 4.15-fold increase in WT sKL versus Null mice ($p_{adj} = 0.06$) and a 6-fold increase in SOD1^{G93A} sKL compared to SOD1^{G93A} Null ($p_{adj} = 0.005$).

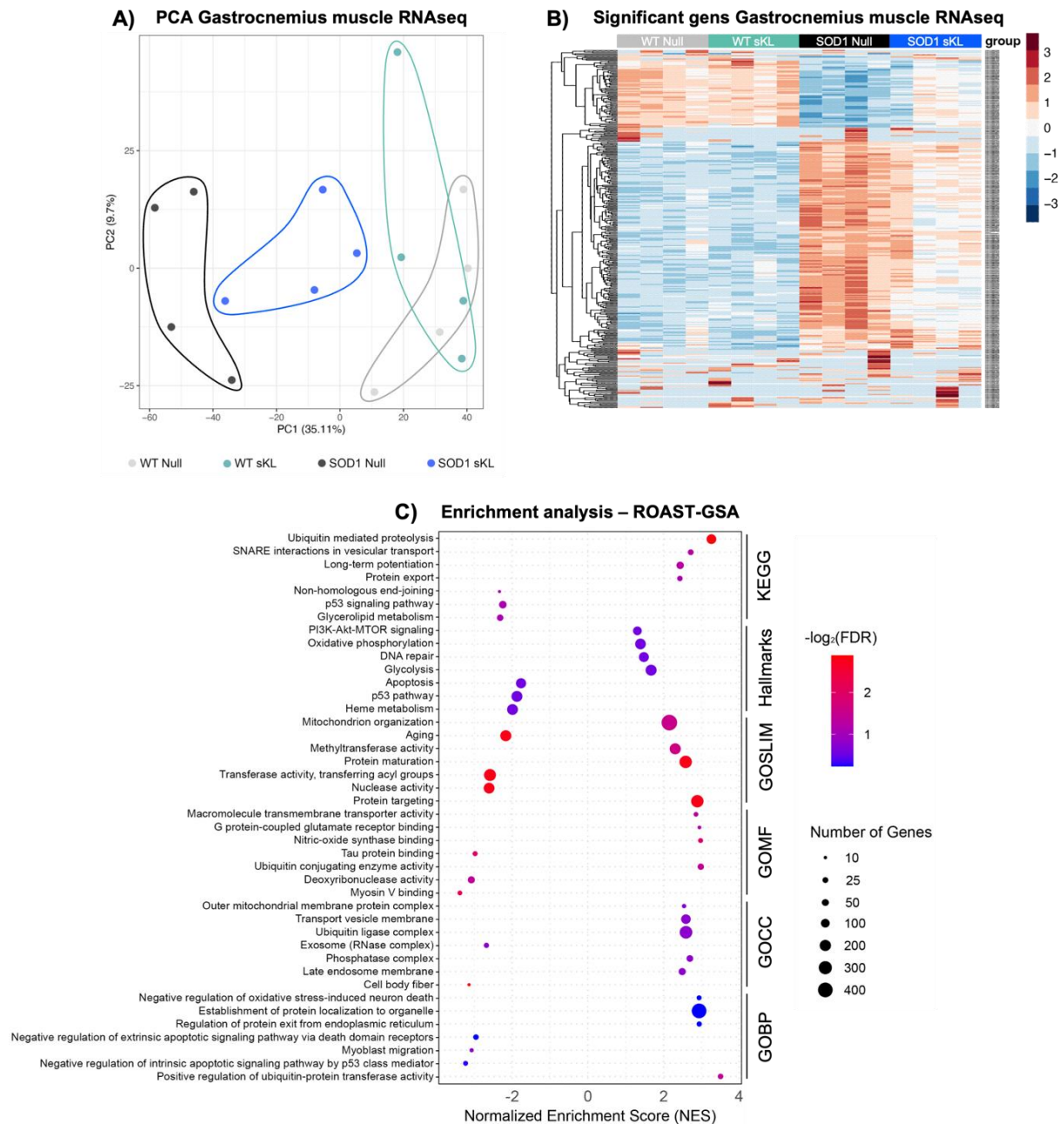


Figure 46. Profiling the gastrocnemius muscle transcriptome of WT and SOD1^{G93A} female mice treated with MyoAAV-Null and sKL vectors. (A) Conducting principal component analysis (PCA) on RNAseq data illustrates three distinct patterns of gene expression. (B) Heatmap indicating the genes that are significantly differentially expressed across the four groups, with each column representing a particular animal ($n = 4/\text{group}$) and each row an upregulated (red) or downregulated (blue) gene. (C) Dot plot of pathway enrichment analysis highlighting differences in representative KEGG, Hallmarks, and GO terms between SOD1 mice treated with MyoAAV-sKL and those treated with MyoAAV-Null.

GSEA was then used to determine whether predefined sets of genes showed statistically significant, concordant differences between sKL and Null-treated SOD1^{G93A} mice. However, in contrast to observations in the spinal cord, genesets did not reach significant enrichment (FDR < 0.1) in either condition after adjusting for multiple tests.

Nevertheless, we still plotted the most representative GO, Hallmark, and KEGG categories relevant to sKL overexpression, muscle, and ALS biology, as illustrated in Figure 45C. This emphasis was placed on those with the highest enrichment, as numerous individual genes within these ontology families exhibited significant differences. Among the functional terms that exhibited the most positive NES, the KEGG pathway related to **ubiquitin-mediated proteolysis** showed significant upregulation of the expression of ubiquitin-conjugating enzymes and E3 ubiquitin ligases (Fig. 47A).

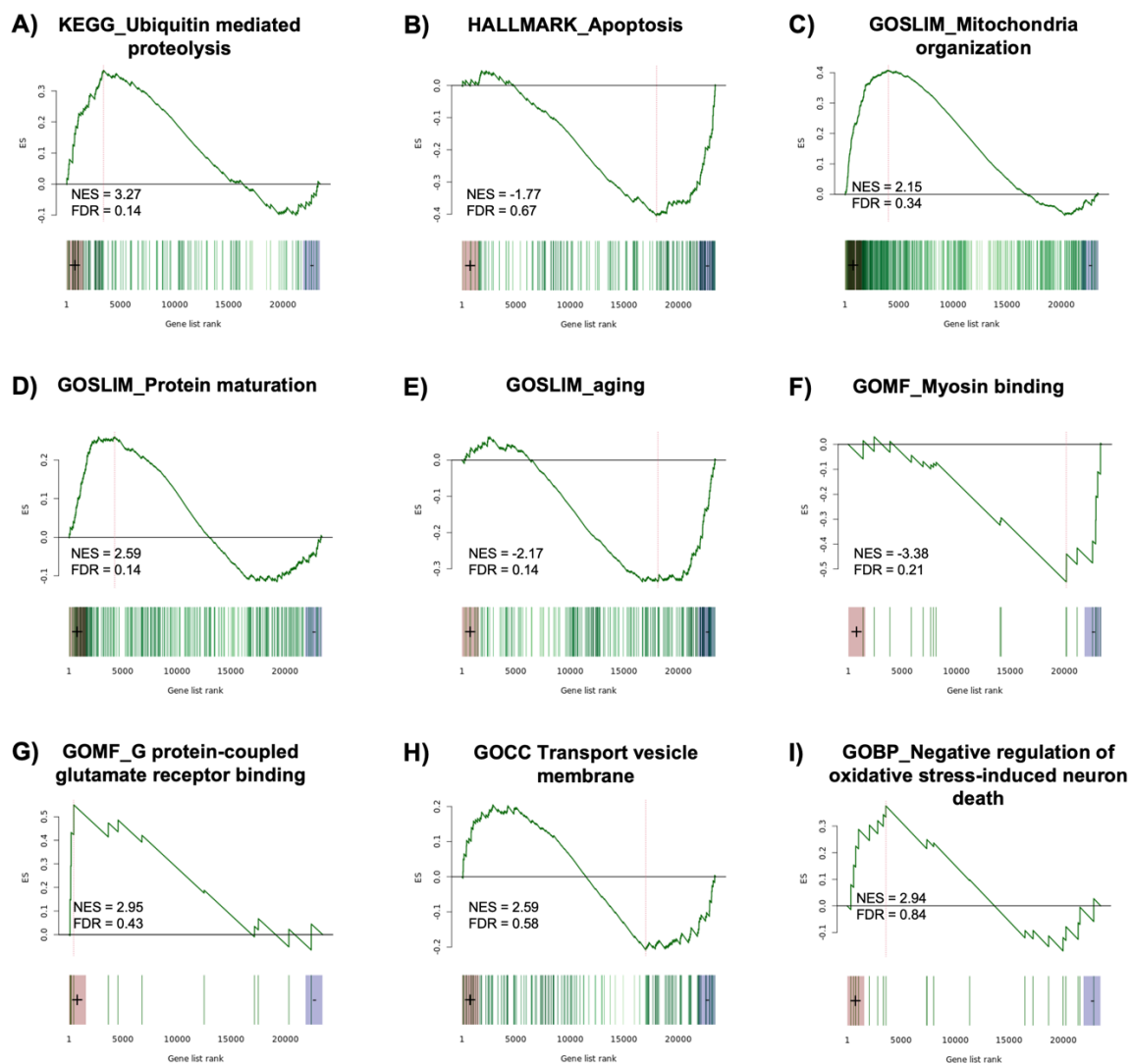


Figure 47. GSEA portrays connections between genes and biological concepts in the gastrocnemius muscle. Downregulated and upregulated GSEA of representative KEGG (A), Hallmark (B), GOSLIM (C, D, E), GOMF (F, G), GOCC (H), and GOBP (I) terms that exhibit biological significance in SOD1^{G93A} female mice when sKL is overexpressed in this muscle. Genes from a set that tend to cluster at the top (positive NES) of the gene list are enriched in SOD1^{G93A} sKL mice, whereas those at the bottom (negative NES) are predominant in the SOD1^{G93A} Null group.

In contrast, the expression of genes associated with **apoptosis** (*Casp3*, *Bcl2l1* and *Cdkn1a*) and **aging** (*App*, *Dkk1*, *Tsc2* and *Ypel2*) was significantly downregulated in sKL-overexpressing muscles compared to null muscles from SOD1^{G93A} mice (Fig. 47B, E).

Numerous genes involved in **mitochondrial structure and function**, such as *Tomm*, *Timm*, and *Tmem* transporters, *Vdac1* channel, cytochrome c oxidase, and *Sod2*, exhibited an enrichment (Fig. 47C), whereas *Mmp9* was significantly downregulated. The GO term "myosin V binding" showed a particularly low NES (Fig. 47F), implicating various proteins involved in the **trafficking of organelles**, vesicles, receptors, and RNA. In particular, the expression of Rab GTPases and the ALS-related gene *Fus* showed a significant negative fold change due to the therapy. Conversely, genes related to the **binding of G-protein-coupled glutamate receptors** (mainly from the calmodulin and Homer families) and those involved in **coping with oxidative stress-induced neuron death** (Thioredoxin-related proteins, *Selenof*, *Nqp1*, *Nnt*, *Park7*, *Oxr1*, *Il-10*, *Atf4*, and *Atg7*) demonstrated increased expression (Fig. 47G, I). Finally, structural genes (myosin-related) and genes relevant to the maintenance of muscle integrity and function (*Ankrd1*, *Col19a1*, *Sln*, and *Snx10*) as well as those with roles in myogenesis (*Myod1*, *Myog*, *Zfp697* or *Rrad*) exhibited notable alterations in diseased mice, but were partially corrected by the treatment.

4.2.2. Proteomic analysis of the gastrocnemius muscle in sKL- and Null-treated mice

To confirm RNAseq results, proteomic studies were conducted on the contralateral gastrocnemius muscle of the same group of animals. Mass spectrometry initially identified a total of 4,316 protein groups, a number that was reduced to 3,284 by excluding protein groups identified only by a unique peptide or those with missing values in any of the four conditions. We performed a differential abundance analysis of the four groups with the contrasts outlined in Table 6.

Table 6. Numbers of neutral proteins and proteins significantly over- or underabundant in the gastrocnemius muscle in each contrast ($p_{adj} < 0.05$; neutral = $-1.5 < FC < 1.5$, up = $FC > 1.5$, down = $FC < -1.5$).

Contrast	Down	Neutral	Up
SOD1 sKL_SOD1 Null	73	3084	107
SOD1 Null_WT Null	157	2726	360
SOD1 Null_WT sKL	175	2680	389
SOD1 sKL_WT Null	0	3078	9
SOD1 sKL_WT sKL	1	3064	11
WT sKL_WT Null	0	2947	0

SOD1^{G93A} mice showed 157 underabundant and 360 overabundant proteins in the gastrocnemius muscle compared to WT Null mice, which was corrected by the treatment to no underabundant proteins and only 9 overabundant proteins. When comparing SOD1^{G93A} mice, the overexpression of sKL in the gastrocnemius muscle resulted in 107 upregulated and 73 downregulated proteins (Fig. 48B). Of note, the KL protein was not among the overabundant hits as it was not detected by LC-MS/MS, probably due to its highly glycosylated state, which complicates detection (Pan et al., 2011).

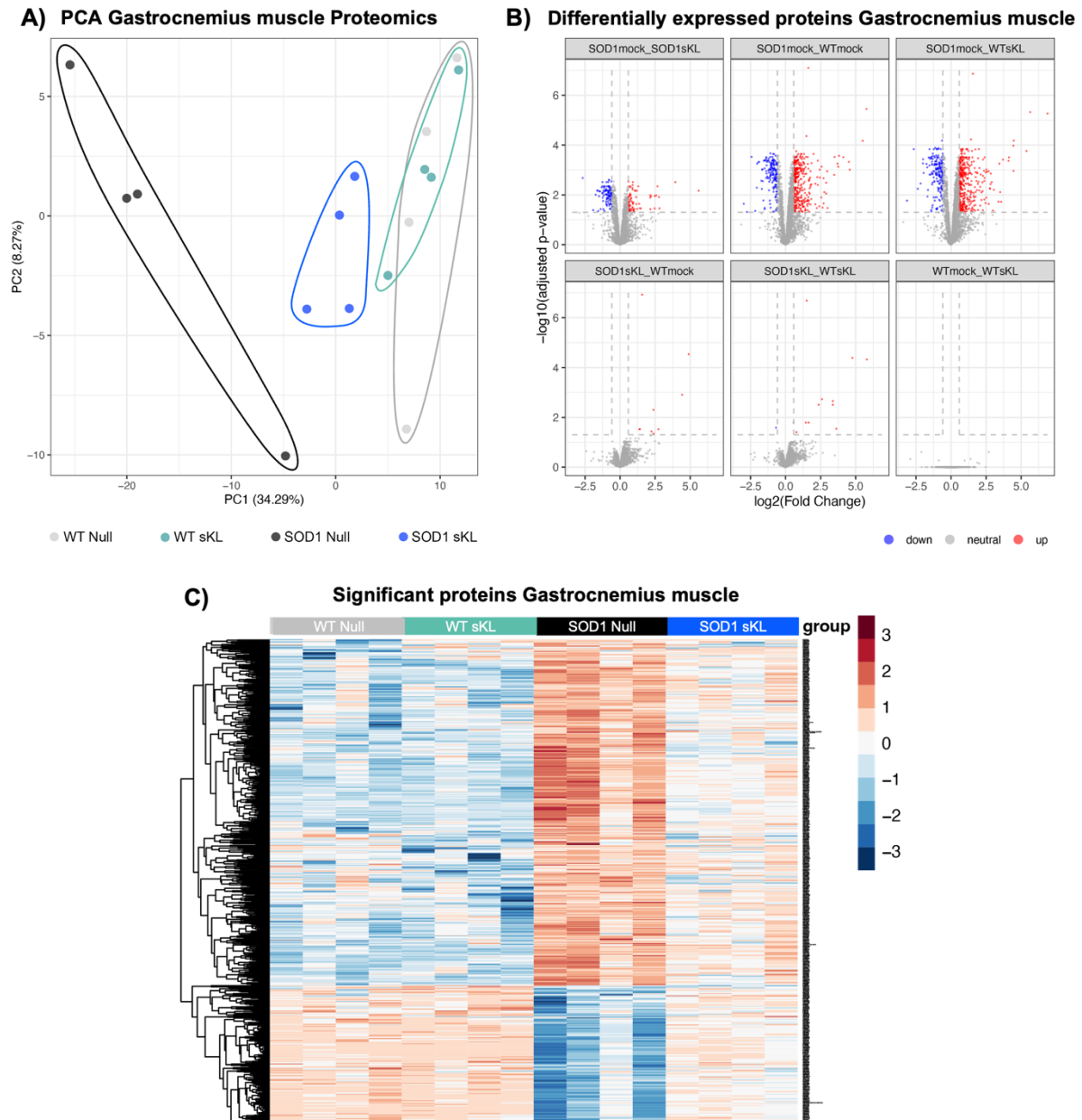


Figure 48. Proteomic analysis of gastrocnemius muscle in female WT and SOD1^{G93A} mice treated with MyoAAV or sKL vectors. (A) Principal component analysis (PCA) of proteomic data reveals three distinct expression patterns in the four biological groups. (B) Construction of Volcano plots to show significant proteins comparing the different conditions, with upregulated proteins in red and downregulated proteins in blue ($p_{adj} < 0.05$, fold change $> |1.5|$). (C) Heatmap of significant proteins in the four groups, with each column corresponding to a specific animal in the same order as in the RNAseq data ($n = 4$).

The clustering heatmap of significant proteins ($|FC| > 1.5$ and $p_{adj} < 0.05$) in any of the six contrasts shows two main protein clusters. The first cluster with highly abundant proteins in the SOD1^{G93A} Null group, which are slightly lower in the SOD1^{G93A} sKL group and have the lowest abundance in the WT groups. The second with the lowest abundance in the SOD1^{G93A} Null samples and similar abundance in the WT and SOD1^{G93A} sKL groups. PCA clearly shows this correction in the protein expression patterns, with the SOD1^{G93A} sKL cluster closer to the WT groups than to the SOD1^{G93A} Null group (Fig. 48A). Given these observations, the treatment with sKL appears to partially reverse the effects of hSOD1^{G93A} in the muscle, particularly when the proteins are underexpressed in the null condition.

Next, we examined the expression levels of the top 20 proteins with biological significance. These proteins were either underabundant or overabundant in the gastrocnemius muscle of SOD1^{G93A} Null mice, and their expression profile was reverted by the treatment (Fig. 49A, B). As expected, the direct overexpression of sKL in this tissue led to a more pronounced correction of the dysregulated proteins than in the spinal cord.

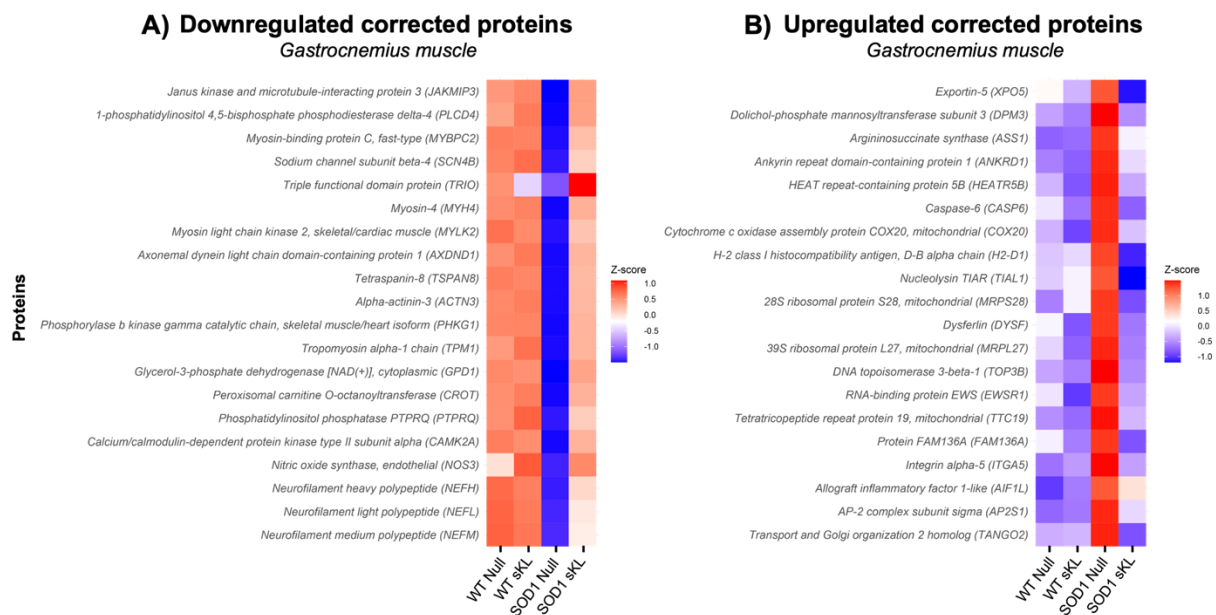


Figure 49. Heatmap illustrating corrected proteins in the gastrocnemius muscle of SOD1^{G93A} female mice treated with sKL. (A) Shows top downregulated proteins, while (B) highlights top upregulated proteins in the gastrocnemius muscle of SOD1^{G93A} mice that were corrected by the sKL overexpression.

Most of the downregulated and corrected proteins belong to functions related to **microtubule and cytoskeletal dynamics**. They are myosin chains (MYH4), myosin kinases (MYLK2), myosin- or actin-binding proteins (MYBPC2, TRIO, ACTN3) or cross-linkers of actin and myosin (TPM1), which is consistent with the RNAseq results. The downregulation of neurofilament polypeptides in the muscle of SOD1^{G93A} mice is worth mentioning as they are critical for motor axons, and this is partially corrected by the treatment.

TRIO and AXDND1 are proteins associated with axon pathfinding and neurite outgrowth, respectively, and their expression is markedly increased in response to sKL. On the other hand, expression of proteins related to **muscle damage and integrity**, such as ANKRD1, COL19A1, and RRAD, was heavily upregulated in SOD1^{G93A} null mice and notably corrected by the treatment (6.9-, 2.7- and 2.15-fold change decrease, respectively). Fibrotic markers indicative of atrophy were downregulated by enhancing sKL levels in SOD1^{G93A} muscles, including several collagen proteins (COL1A1, COL2A1, COL8A1, COL4A2, COL4A3), microfibril-associated glycoprotein 4 (MFAP4), Igfn1 and TGF- β .

Several other proteins upregulated by sKL are involved in **survival, signal transduction, and synaptic plasticity** (JAKMIP3, PLCD4, TSPAN8, PTPRQ, and CAMK2A), while the pro-apoptotic proteins caspase-6 and caspase-3 are downregulated. Other dysregulated proteins are involved in **metabolism** and potentially influence **energy supply** (NNT, PHKG1, GDPH, ALDH1L2, HIG1, ASS1).

As observed in the spinal cord, we also found a correction of the overabundance of various **RNA trafficking** and **processing proteins** in the muscle. These include exportin-5, TIAL1, ribosomal proteins, and the RNA-binding proteins FUS, EWSR1, hRNPA2B1, hnRNPF, hnRNPU, hnRNPLL, ILF3 or SRSF3. While not being among the top upregulated proteins, numerous proteins belonging to the ubiquitin-ligase family (Parkin7, E3A, E3B, ARIH1, ARIH2, UBR4, HERC2, RNF123, WWP1, and RNF14), ubiquitin conjugation factors and ubiquitin-conjugating enzymes (E2 D1, E2 H, E2 K, E2 L3, E2 R, E4A, and UBC12) demonstrated a significant positive fold-change, thereby affecting protein turnover. Contrarily, ubiquitin C-terminal hydrolase L1 (UCH-L1), which has the opposite function, displayed a 2.65-fold decrease in its expression. The inclusion-forming ubiquitin-binding protein 62 (p62), also displayed a significant reduction in the muscles of sKL-treated SOD1^{G93A} animals.

The elevated levels of H-2 class I histocompatibility antigen, allograft inflammatory factor 1-like (also known as Iba1), tapasin, or galectin-3 indicate an **inflammatory** state in the muscles, which is in part alleviated by the overexpression of sKL. The role of the remaining altered proteins involves various mechanisms relevant to **intracellular transport** and **vesicle trafficking** (AP2S1, TANGO2, and DYSF), **excitability** (sodium channel subunit SCN4B), **vascular regulation** (NOS3), **glycosylation and protein-protein interactions** (DPM3, HEATR5B, and TTC19), **mitochondrial dysfunction** (COX20, LIAS, MCU, MICU or Slc25a22) and **DNA dynamics and repair** (TOP1, TOP3B).

4.3. Integration of RNAseq and proteomics data in the spinal cord and gastrocnemius muscle

The integration of proteomics and RNAseq analyses on the same samples enables to identification of correlations between mRNA and protein levels and thus a more holistic understanding of biological processes. Differences between RNA and protein levels can highlight post-transcriptional regulatory processes such as translation efficiency, mRNA stability, or post-translational modifications and reveal insights into molecular pathways that may be neglected when analyzing one of the two data sets alone.

To combine the data sets, we compared the p-values ($p < 0.1$) for the top and bottom differentially expressed genes and proteins when evaluating the SOD1^{G93A} sKL group against the SOD1^{G93A} Null group (Fig. 50). In the **spinal cord**, **downregulated pathways** were more important than upregulated pathways in both technologies. In particular, downregulated GO terms that showed a stronger correlation between mRNA and proteins included **stress response to metal ions**, **regulation of membrane permeability**, **extracellular space**, **chemokine binding**, and **INF- α response** (Fig. 50A).

Categories associated with **cytoskeletal binding dynamics** and **RNA-binding proteins** showed a more significant downregulation at the protein level than at the mRNA level. Conversely, pathways associated with apoptosis, mitochondrial changes, membrane permeability, oxidative stress, and organelles showed greater significance at the mRNA level. Interestingly, the integration of RNAseq and proteomic data underscored the significant downregulation of multiple **inflammation-related mechanisms** that were overlooked in the independent analyses.

As for the **upregulated** mechanisms in the **spinal cord** (Fig. 50B), most are associated with structural functions and cellular components. The strongest correlation in functional annotations is observed in **synapse function**, **neurotransmitter** and **ion transport**, **distal axon**, and **neuromuscular process**, which is consistent with the main RNAseq results. At the protein level, functional terms related to **oxidative phosphorylation**, oxidoreductase complex, mitochondrion organization, and the mitochondrion in general show the greatest significance between sKL-treated and untreated SOD1^{G93A} mice.

Other notable upregulated mechanisms include ubiquitin ligase-substrate adaptor activity or SUMMO binding, which is required for **proteolysis**, and flippase activity, which is essential for membrane repair and signal transduction.

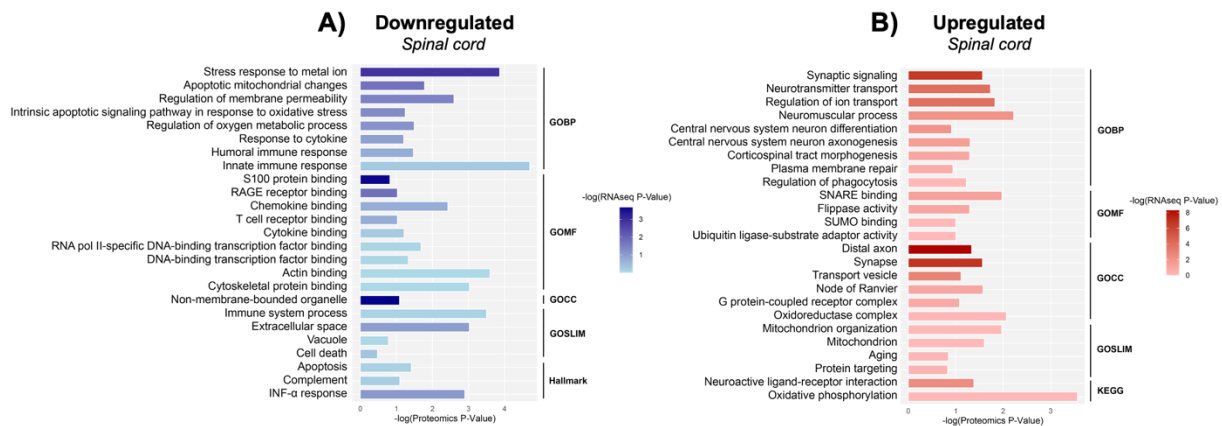


Figure 50. Congruence of RNAseq with proteomic studies in the spinal cord of SOD1^{G93A} female mice. Bar graphs comparing hypergeometric tests of RNAseq and proteomics with their respective p-values for the major down- (A) and upregulated (B) pathways in SOD1^{G93A} sKL-treated mice compared to null controls.

In **gastrocnemius muscles**, fewer upper and lower differentially expressed genes and proteins showed a significant p-value ($p < 0.1$) when SOD1^{G93A} sKL mice were compared with SOD1^{G93A} Null mice (Fig. 51). However, the correlation of **downregulated pathways** between the two technologies was similarly significant. The major downregulated pathways were related to **DNA topological changes** and **nuclear speck formation**. Nuclear specks, i.e. clusters of interchromatin granules, serve as sites for the storage of splicing factors involved in mRNA maturation, export, and RNA metabolism (Galganski et al., 2017). This association is related to the observed decrease in genes and proteins within the GO annotations of mRNA binding, single-stranded RNA binding, and ribonucleoprotein granule (Fig. 51A).

Consistent downregulations at both RNA and protein levels were also observed in the positive regulation of the **inflammatory response**, the negative regulation of macrophage migration, and glial cell projection. The negative regulation of macrophage migration involved the downregulation of genes such as macrophage migration inhibitory factor (MIF), D-dopachrome tautomerase 1 (MIF-2), emilin1, and integrin $\alpha 5$. These are potent pro-inflammatory products of activated macrophages that support macrophage survival and function (Merk et al., 2011; Mitchell et al., 2002). In addition, the antioxidant effect of KL has been confirmed by the inhibition of signaling pathways related to the positive regulation of **ROS biosynthesis, oxidases, or NO-forming activities**.

As already observed in the proteomic analysis, the **upregulated pathways** in the gastrocnemius muscles focused on functions related to **skeletal muscle fiber contraction, muscle development, and differentiation** (Fig. 51B). Another focus was on **glycogen, ribonucleoside, energy reserves** as well as **cysteine and methionine** metabolic processes.

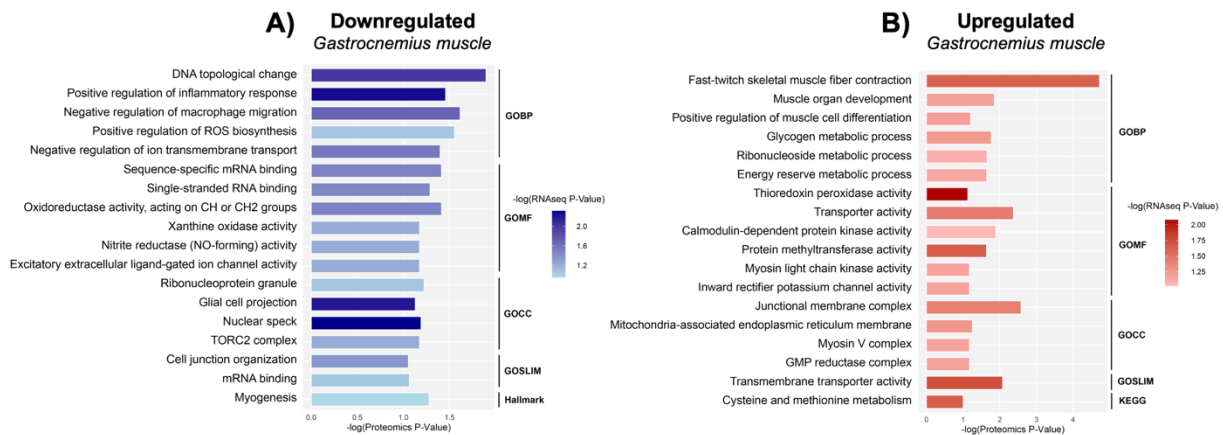


Figure 51. Alignment between RNAseq and proteomic findings in the gastrocnemius muscle of SOD1^{G93A} female mice. Bar charts contrasting hypergeometric tests of RNAseq and proteomics along with their adjusted p-values for prominent down- (A) and upregulated (B) pathways in SOD1^{G93A} mice treated with sKL in comparison to null controls.

Upregulation was found in genes and protein families associated with transporter activities, myosin kinases, and the myosin V complex, which serves as tethers for various cargoes. In particular, overexpression of sKL was found to induce the expression of the **antioxidant** thioredoxin/peroxiredoxin system, as previously reported (Zeldich et al., 2014). The Ca²⁺/calmodulin-dependent protein kinase signaling pathway, which is crucial for **synaptic plasticity** and **muscle contraction**, was also enriched by the overexpression of sKL.

Furthermore, **mitochondria-associated endoplasmic reticulum membranes (MAM)**, which act as an interface between mitochondria and ER, have been linked to ALS pathology in several studies (Bernal et al., 2023). The expression of genes and proteins within these structures, such as TOMM20, TMX2, or ATG5, was increased in the muscles of treated animals.

Overall, transcriptomic and proteomic analyses confirmed the pleiotropic effects of sKL by demonstrating its protective effects in both the spinal cord and muscle, consistent with the functional data presented in this thesis. The integration of these techniques facilitated the identification of molecular pathways obscured by other prominent findings when analyzed independently.

DISCUSSION

The exact trigger for MN degeneration remains still unknown in most cases, despite the extensive research conducted in the field of ALS and other MND. One of the reasons is the diverse genetic and molecular landscape of ALS, which complicates the finding of effective treatments or a cure for this devastating disease. Unfortunately, most promising preclinical research studies have failed to make the transition to the clinic.

Traditionally, clinical trials for ALS have focused primarily on targeting single disease mechanisms or, in certain cases, a few of the primary alterations. This approach often involves the combination of pharmacological drugs intending to achieve complementary effects. However, the complex and heterogeneous nature of ALS has rendered these limited-target strategies ineffective. An emerging perspective advocates for a more synergistic approach and the consideration of targeting multiple pathways. In this context, we proposed the therapeutic exploration of the pleiotropic protein Klotho. The unique property of Klotho is that it can act as a single protein on multiple signaling pathways involved in neurodegeneration. This has the advantage of simplifying therapeutic interventions and bypassing the need for complex combinatorial treatments with inconvenient biosafety profiles.

Expression levels of Klotho in models of MN degeneration

The Klotho gene was first reported in a mouse model with extremely premature aging (Kuro-o et al., 1997). The full gene encodes for a transmembrane protein presenting two extracellular domains and a short intracellular moiety. The secreted form arises from a premature STOP codon before the second extracellular domain and the transmembrane domain (Shiraki-Iida et al., 1998). These isoforms show different spatiotemporal expression patterns, and although sharing most of the tissular expression, the ratio between the two isoforms shifts depending on the tissue. However, most authors do not discriminate between the secreted isoform and the shed forms from the transmembrane isoform, while others doubt active sKL protein translation. In a controversial study, Mencke and colleagues (2017) argued that the premature termination codon primes the human sKL mRNA for degradation by NMD. These authors showed that the alternative KL mRNA was enriched in the NMD core factor UPF1, that it was not associated with polysomes, and that it colocalized with P bodies, where NMD transcripts become degraded. However, upon NMD inhibition there was only a modest increase in sKL transcripts. In Western blot studies using an antibody against the KL1 domain they did detect a 130 KDa and a second 65 KDa band when overexpressing KL in HK-2 cells but, despite having the size of sKL, determined that this second band was residual albumin. Regardless of that, the cDNA sequence of sKL has the potential to produce a stable protein exerting biologically active functions when expressed exogenously (Massó et al., 2018; Roig-Soriano et al., 2022; Roig-Soriano et al., 2023).

Since its discovery, KL has been implicated in several disease conditions, mainly in age-related disorders. Several studies have revealed that KL serum levels are decreased in the general aged population and inflammatory conditions, with a significant decrease in patients with Alzheimer's disease compared to non-pathological aging individuals (Semba et al., 2014; Wu & Chen, 2022). In aging and chronic kidney disease, a decrease in nephron number increases FGF23 expression to maintain phosphate balance, which lowers serum levels of vitamin D, followed by an increase in PTH levels, further increasing FGF23 and resulting in the suppression of KL expression (Isakova et al., 2011). Different studies have also indicated that in the muscle and the cartilage, KL can undergo epigenetic silencing on disease conditions and promote pathology (Iijima et al., 2023; Sahu et al., 2018; Wehling-Henricks, et al., 2016).

In this sense, we sought to explore the endogenous levels of KL in the SOD1^{G93A} mouse model of ALS at the advanced stage of 16 weeks, when MN loss and muscle atrophy are preeminent. The identification of secreted and shed protein forms for KL has been hindered by its low expression in certain tissues in adults and the absence of suitable antibodies for the KL1 domain (Imura et al., 2004; Kato et al., 2000; Xiao et al., 2019). In a prior study conducted by our group, we developed an antibody targeting the exclusive C-terminal tail of sKL (Massó et al., 2015). This antibody exhibited specific detection of sKL in both the brain and kidney through Western blot analysis. In this study, however, we did not succeed in detecting endogenous sKL in the spinal cord or muscles by Western blot, but only by TSA-amplified immunohistochemical analyses in the muscle (Fig. 33A).

Due to these considerations, we directed our efforts towards analyzing the mRNA expression of KL isoforms in the spinal cord, motor cortex, sciatic nerve, muscles, and kidney samples from SOD1^{G93A} mice, as previous studies have demonstrated that KL is mainly regulated at the epigenetic level. sKL expression levels were highly downregulated in the two muscles analyzed, indicating a preferential role for this isoform in these tissues (Fig. 12A). This is in accordance with previous studies showing that KL loss severely impairs muscle progenitor stem cells (MPCs) function *in vitro* and during muscle regeneration *in vivo*, and further demonstrated that supplementation with the sKL isoform rejuvenated aged MPCs (Sahu et al., 2018). A subsequent study identified the secreted form as the most differentially upregulated isoform upon muscle injury (Welc et al., 2020). Of course, it remains elusive whether the decrease in sKL in SOD1^{G93A} muscles is only associated with muscle atrophy or it could be as well associated with muscle denervation in ALS.

On the other hand, mKL expression was significantly decreased in the nervous tissues analyzed. In mice, mKL is the predominant transcript in the CNS, where it is mainly involved in synaptic activity and plasticity, enhancing cognition and memory formation (Castner et al., 2023; Dubal et

al., 2015; Roig - Soriano et al., 2022). Interestingly, mKL levels only showed a significant reduction in the gastrocnemius muscle but not in the soleus muscle (Fig. 12B). The soleus muscle is a slow-twitching muscle known to be ALS-resistant and becomes denervated and degenerates at the end stage of the disease (Hegedus et al., 2007). Additionally, the kidney, a primary site for the production of processed circulating forms of KL that may compensate for tissue deficiencies, displayed a tendency toward reduced mKL levels. This suggests that alterations in mKL expression are not confined to primary tissues affected by the disease.

To investigate whether the decline in KL expression was caused by SOD1^{G93A} aggregation or is a consequence of MN degeneration and muscle atrophy we assessed the levels in the BPI model. In the forelimb muscles, the expression of both isoforms correlated with the days-post injury, and therefore with the degree of atrophy (Fig. 12D, F). In the spinal cord, sKL levels significantly decreased after the injury and were partially restored at 28 dpi, indicating a temporal downregulation during the initial stages, where inflammation is prominent (Quintão et al., 2006). In contrast, mKL levels remain unchanged after BPI, contrary to what was observed in SOD1^{G93A} mice, suggesting that its downregulation may be more related to ALS mechanisms (Fig. 12E). Although, it must be considered that in nerve injuries MN loss is considerably lower than in the SOD1^{G93A} model, especially in adult mice and distal axotomies (Liu & Wang, 2020). Taken together, these results suggest that the expression of KL is silenced as a consequence of the neurodegeneration and muscle atrophy that occurs in ALS.

To continue studying KL alterations in ALS we have initiated procedures to gather samples from patients diagnosed with spinal-onset ALS. Considering that sKL levels are very low in mice but considerable in humans (Matsumura et al., 1998; Shiraki-Iida et al., 1998), we plan to analyze its levels in the serum, CSF, and muscle punch biopsies from patients.

Klotho protection of MN in spinal cord organotypic cultures exposed to glutamate

Various *in vitro* models can be employed to investigate glutamate neurotoxicity, with one example being the SCOCs. The advantages of utilizing rat-cultured spinal cord slices include the assessment of MN degeneration and the exploration of potential therapeutic in a close-to *in vivo* cytoarchitecture (Herrando-Grabulosa et al., 2016). Importantly, rat explants eliminate the necessity for introducing growth factors or external stimuli, which could otherwise serve as confounding variables. This particular model was initially developed to selectively inhibit glutamate transport, leading to a gradual increase in glutamate concentration in the culture medium (Rothstein et al., 1993).

We have modified this system by directly adding glutamate to the cultured sections, causing an acute exposure that leads to the fast degeneration of spinal MN over the following days (Guzmán-Lenis et al., 2009).

To investigate the effect of KL in mitigating the excitotoxicity, we generated vectors containing coding sequences for sKL, mKL, and a null sequence under the CMV promoter. This approach allows the study of the sKL isoform, as only the pKL form is commercially available, as well as the transmembrane form, which cannot be evaluated otherwise. However, its application is limited to a preemptive strategy, as glutamate triggers rapid MN death, and the vectors require a certain period to express the transgene, unlike recombinant proteins that can be concomitantly supplemented to the media. In a previous study, adenoviral vectors were microinjected directly into the sections, resulting in focal ectopic expression of the transgene (Pasterkamp et al., 2000). However, we dismissed this invasive approach, which may induce further MN death and glial reactivity. Instead, we tested the addition of Ad-GFP particles to the media as described for other types of organotypic sections (Baker et al., 1998; Ridoux et al., 1995), and the application of a droplet to the slice. The addition of a one-microliter droplet showed a higher number of transduced cells than the dilution of the viral preparations in the media (Fig. 13B).

Adenoviral vectors are known for their higher infectivity *in vitro* compared to AAV vectors (Kremer & Perricaudet, 1995), whereas AAV vectors are widely used for *in vivo* applications. We sought to concurrently evaluate Ad and AAV vectors encoding GFP, as these explants are more likely to behave as a tissue than a cell culture. AAV vectors exhibited superior transduction, reaching a greater number of neurons, and a tendency to transduce more astrocytes (Fig. 14). Nevertheless, most cells did not co-localize with SMI-32, GFAP, or Iba1, suggesting that the transduction primarily targeted GFAP⁻ and Iba⁻ glial cells. Based on the morphology of the transduced cells, oligodendrocytes could be the primary target of Ad and AAV vectors in these cultures, though additional co-labeling studies are required to confirm the specific tropism to this cell type.

The preventive overexpression of sKL and mKL mediated by AAV9 vectors resulted in complete protection from glutamate-induced neuronal death (Fig. 15B, D). The results indicate that this effect is mediated by the soluble forms of KL, as most of the neurons are not transduced by viral vectors, more precisely by the KL1 domain found in both isoforms. AAV9-CMV-mKL vectors showed a tendency for a less extensive infection of the sections or did not avoid the inherent death of the glia, compared to AAV9-CMV-sKL vectors. However, the mKL levels achieved within the sections were enough to provide neuronal protection despite not protecting transduced KL⁺ glial cells (Fig. 15 C, E). The exposure to glutamate induces a rapid influx of calcium, and MNs exhibit limited buffering of the resulting cytosolic calcium overload (Lips & Keller, 1998). Consequently, a substantial amount of calcium is taken up into mitochondria, leading to a pronounced generation

of ROS (Carriedo et al., 2000). ROS, in turn, disrupt glutamate transport in neighboring astrocytes and propagate the injury cascade (Rao et al., 2003). As previously reported, KL may confer this protection against glutamate by enhancing resistance to ROS generated during the insult (Zeldich et al., 2014). This protection is achieved through the activation of the antioxidant system Trx-Prx and the ASK1/p38 MAPK pathway. Furthermore, in these cultures TNF- α was described to potentiate MN death and cooperate in the induction of oxidative stress. KL is a potent inhibitor of NF- κ B signaling that could be abrogating glutamate-mediated MN death induced by TNF- α (Tolosa et al., 2011), although we did not check for it.

Additional *in vitro* studies on iPSC-derived MNs from different ALS patients will be performed in order to assess the effect of KL on different ALS backgrounds.

Gene therapy targeting sKL overexpression to the spinal cord

The promising *in vitro* results encouraged us to move into treating the SOD1^{G93A} mouse model of ALS. Recent studies on KL, conducted by us and others in various mouse models of neurodegenerative diseases, suggested that KL had the potential to mitigate the pathological progression of ALS (Zeldich et al., 2019). KL could achieve this by mitigating oxidative stress (Kurosu et al., 2005), promoting neuronal survival (Zeldich et al., 2014), enhancing oligodendrocyte function (Chen et al., 2013; Zeldich et al., 2015), reducing senescence (Roig - Soriano et al., 2022) and the proinflammatory immune response (Guo et al., 2018).

The first approach we tested was intrathecal administration of AAV9-CAG-sKL vectors to achieve extensive spinal cord transduction. We chose to overexpress sKL instead of mKL for three reasons. First, we have previously shown that high circulating levels of pKL, but not sKL, have deleterious effects on mineral metabolism and bone microstructure. sKL did not significantly alter phosphate and calcium metabolism, demonstrating that the KL2 domain found in pKL and mKL is necessary for these noxious effects (Roig-Soriano et al., 2023). Second, we observed that the number of transduced glial cells expressing mKL decreased when SCOCs were exposed to glutamate, whereas this was not the case for sKL. Third, given the hormonal effect of KL in protecting MNs, we reasoned that sKL would be more readily available and present in higher amounts than mKL, which remains in the membrane and must be proteolyzed to release the KL1 domain.

The overexpression of sKL in the spinal cord only showed significant improvements in neuromuscular function in SOD1^{G93A} females for the TA muscles. The PL muscles of the paw showed a tendency for preservation that was not significant (Fig. 17A, C). The PL muscles are more distal than the TA muscles, and altered axon excitability and EMG abnormalities in ALS

patients occur in a distal to proximal fashion (Nakata et al., 2006; Shayya et al., 2018), indicating that sKL treatment may be able to preserve proximal but not distal muscle function. In males, no improvements were noted in the CMAP amplitudes; nevertheless, it is important to note that this study involved a limited number of animals, and additional research is required to validate these findings. Regarding the MEPs, we observed a strong preservation of their amplitude again in females but not in males (Fig. 18). Despite the outcomes in neuromuscular function, both treated males and females exhibited an improved performance in the rotarod test (Fig. 19).

SOD1^{G93A} mice have gender-related differences that lead to more aggressive disease phenotypes in males, potentially accounting for their lack of response to the treatment. SOD1^{G93A} females display higher amplitudes of the CMAP compared to age-matched males, and show a delayed onset and longer lifespan (Mancuso et al., 2012; Pfohl et al., 2015). In humans, the incidence of ALS is higher in males than in females. Men generally experience the onset of the disease at a younger age, predominantly in the spinal regions, while women have onset more often in the bulbar region (McCombe & Henderson, 2010). Concerning this matter, a previous report that generated a SOD1^{G93A} mouse model expressing the KL gene ubiquitously from birth also reported more favorable outcomes in females (Zeldich et al., 2019). Multiple studies align with this sexually dimorphic response to potential therapeutics in the SOD1^{G93A} model (Bame et al., 2012; Bankole et al., 2022; Torres et al., 2020; Veldink et al., 2003). Curiously, a study assessing the therapeutic effect of methionine sulfoximine in SOD1^{G93A} animals, demonstrated that castration or ovariectomy neutralized these sex discrepancies on survival and neuromuscular decline, implying that estrogen receptors contribute to the progression of disease (Bame et al., 2012).

In earlier work, we intrathecally administered vectors to SOD1^{G93A} mice coding for the growth factor Nrg1-III and also observed gender-specific beneficial effects (Mòdol-Caballero et al., 2020a). In contrast, when overexpressing Nrg1-I in skeletal muscles, a general correction was observed in both sexes (Mòdol-Caballero et al., 2020b), and this was not further improved when we combined both routes of administration (Mòdol-Caballero et al., 2021b). This previous knowledge, together with the limited outcomes observed in intrathecal gene therapy (Fig. 52), motivated us to shift and explore the potential benefits of boosting sKL levels in the muscles of SOD1^{G93A} mice.

Gene therapy targeting sKL overexpression to the skeletal muscles

A growing body of studies supports the concept that ALS is characterized by a dying-back process, starting with a distal axonopathy (Fischer et al., 2004). Neuromuscular denervation starts much earlier than MN death, thus solely preserving MN soma is insufficient to rescue mSOD1

mice. Axons cannot reestablish connections at the NMJ once they have undergone significant retraction (Dewil et al., 2007; Gould et al., 2006; Ruegsegger et al., 2016). Early abnormalities are detected within muscles in ALS, implicated in the disassembly of NMJ (Bruneteau et al., 2015; Jokic et al., 2005). To target the neuromuscular axis, we considered overexpressing sKL in skeletal muscles throughout the animal's body. This approach aimed to exploit sKL myoregenerative effects within the muscle and its neuroprotective paracrine effect on MN terminals, thus preventing NMJ detachment.

The first approach involved the use of AAV8 vectors administered systemically and the human desmin promoter. This allowed us to restrict expression to striated muscles and avoid off-target effects in other tissues, which was a viable strategy employed in previous works (Mòdol-Caballero, 2020b, 2021a, 2021b). Two groups of female SOD1^{G93A} mice received different intravenous doses of AAV8-Des-sKL vectors, 1.8×10^{14} and 3×10^{14} vg/kg, and were monitored until 16 weeks of age. The lower dose showed no improvements between the treated and null SOD1^{G93A} mice in CMAP amplitudes in the PL and TA muscles. It only demonstrated preservation of MEP at 8 weeks of age, resulting in no significant improvements in rotarod test performance at end stage or in disease onset (Fig. 23B). In contrast, the higher dose of AAV8-Des-sKL significantly preserved the CMAP amplitudes of PL and TA muscles, as well as the MEP up to 12 weeks of age (Fig. 22). Since hind limb deficits are one of the earliest signs of the disease, we incorporated the grip strength test in this second group of animals treated with the highest dose. The grip strength of the animals, as well as the execution of the rotarod task, were significantly preserved until the end stage compared to null-treated SOD1^{G93A} mice (Fig. 23B, D). These data suggest the presence of therapeutic threshold levels for sKL in the muscles, that are only reached by the higher dose of AAV8 vectors.

The overexpression of sKL in the muscles did not improve the loss of body weight in SOD1^{G93A} mice at the lower dose, whereas at the higher dose, it remained curiously lower than in the animals treated with a dose of 1.8×10^{14} vg/kg of null vectors (Fig. 23A). This suggests that either the high dose used, the increased expression of sKL or a combination of both had an impact on the body weight. Although the KL-OE mouse model originally described by Kurosu et al. (2005) showed no differences in growth, weight maintenance, or food intake, subsequent studies have found lower weight gain (Roig-Soriano et al., 2022; Zeldich et al., 2019). A possible explanation could lie in the differences between the strains, with the C3H background being used in the first study and the C57BL/6 background in the others.

Given that KL inhibits insulin and IGF-1 signaling pathways, possibly resembling a calorie restriction model, it is logical to expect KL-OE mice to have lower body weight (Kuro-O et al., 1997; Kurosu et al., 2005). Several authors described KL as an emerging exerkin, with its endogenous levels increasing after chronic exercise and correlating with weight loss (Collins et al., 2023;

Corrêa et al., 2022; Landry et al., 2021). Supplementation with KL reduces adiposity, increases lean mass, and elevates energy expenditure (Rao et al., 2019). Therefore, the effect of sKL overexpression on the maintenance of muscle mass in the treated mice is even more meaningful (Fig. 23E).

Overexpression of sKL in muscles had a remarkable effect on the protection of NMJ and MN in this mouse model (Fig. 24), which explains the observed attenuation of disease progression and preservation of motor function. MN degeneration in SOD1^{G93A} mice is a non-cell autonomous process, with glia playing an important role. Solely restricting the expression of mSOD1 to MNs does not cause disease (Pramatarova et al., 2001) or selectively silencing the mutated gene does not prevent it (Boillée et al., 2006; Ralph et al., 2005). The ablation of mSOD1 from microglia (Boillée et al., 2006), astrocytes (Yamanaka et al., 2008), or oligodendrocytes (Kang et al., 2013) prolongs survival by slowing disease progression. To detect changes in neuroinflammation in the lumbar spinal cord, we performed immunohistochemical analysis at the end stage when remarkable glial reactivity and MN degeneration are present. sKL secretion from the muscles decreased the number and the activation of Iba1⁺ microglial cells observed in SOD1^{G93A} null animals (Fig. 25A, B).

Our results are consistent with the attenuation of proinflammatory responses observed in pKL-supplemented primary microglia from SOD1^{G93A} mice and in microglia from SOD1^{G93A} KL-OE mice, as well as in the SOD1^{G93A} KL-OE bigenic mouse model itself (Zeldich et al., 2019). Remarkably, these authors observed how the *in vitro* application of pKL to SOD1^{G93A} microglia led only to a slight reduction in the production of proinflammatory cytokines, compared to SOD1^{G93A} KL-OE microglia. This finding implies the potential involvement of the secreted form of KL or suggests that the levels of pKL and the duration of its exposure may not align with the conditions of endogenously overexpressed KL in mice. These findings are further corroborated by a study demonstrating that selective knockdown of KL in the choroid plexus led to a general rise in the expression of pro-inflammatory factors, triggered infiltration of macrophages, and increased microglial sensitivity to inflammatory stimuli after LPS exposure. The study also indicated that choroid plexus-produced KL plays a role in regulating the brain-immune system junction (Zhu et al., 2018).

In addition, as detected by GFAP, astrogliosis was significantly reversed near to WT levels (Fig. 25C-F). Likewise, the treatment resulted in the suppression of the fibrotic marker vimentin. The correction in these outcomes was not achieved in SOD1^{G93A} KL-OE bigenic mice (Zeldich et al., 2019). These findings are remarkable, as the secretion of KL by muscles appears to exert influence not only on MNs directly connected through their terminals to muscles but also on glial cells located within the spinal cord. This effect might arise from reduced MN death, considering the local expression of sKL in the muscles. However, given the substantial correction achieved and

the fact that glial cells also accumulate mSOD1, we cannot discard that sKL may be taken up through retrograde axonal transport or activate anti-inflammatory second-order mediators in MN, thereby directly or indirectly mitigating neuroinflammation.

The major limitation of our therapeutic strategy was the use of high doses of AAV8 vectors, which required high manufacturing effort and cost. This constraint also limited the number of animals that could be treated and explains why we did not test this approach in males. Male mice usually have a higher weight than female mice. To achieve the targeted dose of 3×10^{14} vg/kg in males, the viral preparations exceeded the maximum possible volume for injection into the tail vein, unless they were concentrated beforehand. These doses also limited the implementation of our therapeutic strategy, as such high doses approach the upper limits tested in clinical situations. Systemic AAV doses exceeding 5×10^{13} vg/kg in humans and NHPs are associated with the risk of thrombotic microangiopathy (TMA), thrombocytopenia, endothelial injury, hepatotoxicity, DRG toxicity, and fatal lung, kidney, or liver failure. Most patients typically recover from these occurrences with the administration of immune-modulating drugs, though fatalities have been documented in several cases (Arjomandnejad et al., 2023; D. Duan, 2023).

Given this situation, we decided to test our strategy with the recently described MyoAAV capsid (Tabebordbar et al., 2021), aiming to lower the therapeutic dose. MyoAAV vectors were designed to have an increased tropism for skeletal muscles compared to naturally occurring AAV. For comparison with the previous results, we first tested the same construct encapsulated within MyoAAV capsids at three doses in SOD1^{G93A} females: 2×10^{12} , 8×10^{12} , and 1.6×10^{13} vg/kg. The CMAP amplitude exhibited a notable dose-response effect for both muscles (Fig. 27), where the highest dose of 1.6×10^{13} vg/kg had a comparable correction to AAV8 vectors at the 3×10^{14} vg/kg dose. While this correlation was less evident for MEP amplitudes, the highest dose still demonstrated the most substantial preservation. Thus, the dose of 1.6×10^{13} vg/kg for MyoAAV-Des-sKL vectors was selected to continue the studies and the treatment of male SOD1^{G93A} mice. This represents an 18.75-fold decrease in the dose required to achieve therapeutic effects. The question of whether increased doses would yield a greater preservation of neuromuscular function, or if a saturation point for the therapeutic effects of sKL has been reached, remains to be addressed in future investigations.

In these studies, we included WT animals treated with MyoAAV-Des-sKL vectors as a biosafety control, to detect possible effects of sKL overexpression in nerve conduction, body weight, motor function, and glial reactivity under non-pathological conditions. CMAP recording of the muscles revealed no differences between Null and sKL WT animals, indicating no alterations in motor units (Fig. 28A, D, Fig. 29A, D, and Fig. 30A, D).

Regarding the MEP, a significant increase in the amplitudes was detected for the first component in female mice (Fig. 28G), but it was not evident for the second component and was not observed in males for either component (Fig. 29G, H).

Regarding the effects of treatment in SOD1^{G93A} mice, after including more female mice in the study, we continue to observe comparable maintenance of CMAP as observed with AAV8 vectors. Minor differences in controls between the two studies can be explained by inter-batch variability, as the animals compared are not littermates born in the same periods. Importantly, the preservation of CMAP at end-stage for both muscles was similar in female SOD1^{G93A} sKL mice, with a nearly 2-fold significant preservation compared to SOD1^{G93A} null mice (Fig. 28). The preservation of MEP in treated female mice showed a tendency to more than double that of the Null injected SOD1^{G93A} mice. Although both untreated and treated male SOD1^{G93A} mice showed a more pronounced decline than females, there was still a twofold preservation of CMAP when comparing the two groups. In contrast, MEPs showed only a tendency towards modest preservation (Fig. 29).

Interestingly, the effect of the treatment on neuromuscular function was already evident at 8 weeks of age, particularly for the TA CMAP and MEP. This was even though the treatment had only been administered two weeks earlier. Following administration, AAV vector-mediated transgene expression begins around 3 days post-injection and takes at least 3 weeks to peak. However, recent studies suggest that this period can be shortened when high doses are administered systemically (Challis et al., 2019; Greig et al., 2023), therefore allowing sKL to protect against the initial stages of muscle denervation.

To quantitatively assess the functional extent of MN preservation and the health of the functioning MU pools, we performed the MUNE technique and calculated the SMUA for the TA muscles. Treatment significantly preserved the number of MU in both female and male SOD1^{G93A} sKL-treated mice compared to the untreated groups (Fig. 30A, D). The amplitude of individual MU showed modest, but still significant, preservation in males, whereas only a trend toward preservation was seen in females (Fig. 30B, E). The frequency distribution of individual MU indicated more units across all sizes, particularly significant in males, even for large MU (Fig. 30C, F). Taken together, this suggests that the treatment partially maintains the total number of MU. However, a reduction in their size and thus in the muscle fibers innervated by individual MNs is still evident.

Surprisingly, sKL-treated SOD1^{G93A} and WT mice did not exhibit lower body weight than untreated mice with this new serotype (Fig 31. A, B). This suggests that alterations in body weight observed with AAV8 vectors are likely due to the high dose administered or that with this vector serum sKL levels are not sufficient to induce weight changes.

Rotarod performance and strength of female SOD1^{G93A} mice treated with MyoAAV-sKL were improved compared to null controls and showed better results than high-dose AAV8 therapy. Male SOD1^{G93A} mice showed faster disease progression, but the treatment was able to significantly preserve locomotor function in these tests (Fig. 31 C-F). In the treadmill test, treated SOD1^{G93A} males performed significantly better than the untreated controls, while females only manifested a tendency to run faster and longer (Fig. 31G, H). This difference could be explained because most of the untreated males could hardly walk properly. Importantly, sKL overexpression in SOD1^{G93A} mice achieved significant preservation in the mass of the gastrocnemius muscle in both sexes, whereas no differences were noted between null and sKL WT animals (Fig. 31I, J).

In addition, the treatment significantly postponed the clinical onset of the disease as determined by the fall-off time on the rotarod (Fig. 32 A, B). However, most SOD1^{G93A} control animals do not show a consistent fall on the rotarod until the 16th week, when they experience a sudden deterioration in their general condition. Thus, the onset of claspings allows earlier detection of hindlimb abnormalities, that may not become apparent on the rotarod until later stages. At week 16, only a minority of SOD1^{G93A}-Null mice were free of hindlimb claspings, while a significant delay in onset was observed in the sKL-treated groups (Fig. 32 C, D).

Importantly, survival analysis revealed a significantly prolonged lifespan in females and more pronounced in SOD1^{G93A} males, induced by the treatment. These findings are interesting as they are consistent with previous reports of KL overexpression (Kurosu et al., 2005), where KL-OE males showed a longer life expectancy compared to females (30% vs 19%). In our study, higher sKL levels were observed in the serum of treated SOD1^{G93A} males compared to females and were nearly undetectable in SOD1^{G93A} Null mice of both sexes. Conversely, female SOD1^{G93A} mice had higher baseline levels in the gastrocnemius muscle than males, even after treatment. Of note, Zeldich and collaborators (2019) achieved a similar increase in survival time in females when crossing KL-OE and SOD1^{G93A} mice, but in males, we achieved a significantly extended lifespan compared to these bigenic animals. Interestingly, in bonobos and humans, male individuals exhibit lower serum KL levels than females which could be related to differences in life expectancy between genders (Behringer et al., 2018; Pedersen et al., 2013; Semba et al., 2011).

Again, the beneficial effects of sKL muscle overexpression are associated with the preservation of spinal MNs and NMJ innervation (Fig. 33C-F) and attenuation of neuroglial reactivity (Fig. 34). This preservation exceeds that achieved with high-dose AAV8-sKL vectors when comparing female SOD1^{G93A} mice. Immunohistochemistry in TA muscles (Fig. 33A, B) and ELISA in gastrocnemius muscles (Fig. 33G) confirmed the overexpression of sKL. Despite muscles being relatively poor secretors to circulation compared to other organs, some of the secreted KL entered the bloodstream (Fig. 33H). sKL levels in the spinal cord exhibited significant variability, posing a

challenge in determining whether MNs uptake sKL through the NMJ or if endogenous concentrations in the tissue remain higher due to the observed improvements (Fig. 33I), as we can probably discard the entrance of muscle-produced sKL through the BBB (Hu et al., 2016; Leon et al., 2017). Importantly, these protective effects extended beyond the spinal cord and muscles. We found significant preservation in the numbers of pyramidal neurons in layer V of the motor cortex (Fig. 35). Although the integrity of the corticospinal tracts remained compromised, as evidenced by the MEP, the rescue of UMNs could account for the trend towards preserving the amplitude of these potentials and confirm the connectivity between upper and lower MNs in treated animals.

Determination of NFL in plasma or CSF has emerged as a promising candidate for evaluating the degree of neurodegeneration (Benatar et al., 2023). Nevertheless, we did not observe any correction of NFL serum levels by the treatment (Fig. 33J). This is probably because most samples showed levels close to saturation of the analytical technique, and we hypothesize that differences between treated and untreated SOD1^{G93A} mice might be detectable at earlier stages with less MN degeneration.

In view of the multiple therapeutic effects of sKL overexpression in SOD1^{G93A} mice, we decided to treat a small group of animals at the later symptomatic stage of 11 weeks. This is crucial for assessing the therapeutic efficacy of the intervention in a stage more accurately mimicking the disease progression observed in humans at the time of diagnosis. Nerve conduction tests evidenced a slowing of the progressive decline in neuromuscular function shortly after treatment for both muscles in both sexes. This effect was more clearly seen when the individual percentage decline in CMAP amplitude was calculated. In females, treatment reduced neuromuscular impairment in the late phase of the disease (Fig. 37). In males, on the other hand, although preservation was observed fast after treatment, there was a functional decline toward untreated levels as the disease progressed (Fig. 38), particularly in TA muscle. MEPs were not significantly preserved by sKL overexpression at this stage, though a tendency was observed in the female cohort. Remarkably, overexpression of sKL in muscles preserved remaining MNs and NMJs, and provided significant improvements in late-stage rotarod performance and overall strength of SOD1^{G93A} animals (Fig. 39).

Finally, in view that sKL concentration was increased not only in the transduced skeletal muscles but also in the serum, we designed an experiment to target a widespread sKL overexpression, primarily in the liver, to ensure a robust secretion into the bloodstream. The objective was to reaffirm the need for a higher concentration of sKL in muscles and ascertain that the observed benefits in neuromuscular function were not mediated by circulating KL. For that, we used AAV9 vectors, which have a widespread but preferential tropism for the liver (Gray et al., 2011), and the

strong ubiquitous CAG promoter. Using this method, we observed similar sKL levels in the gastrocnemius muscles as in null-injected controls. Nevertheless, the overall serum levels more than doubled when compared to mice treated with MyoAAV-Des-sKL. Despite the rise in circulating sKL levels, no improvement was noted in the CMAP amplitudes of the PL and TA muscles or the MEP amplitudes along follow-up. This underscores the need to induce the overexpression of sKL in muscles rather than achieving high blood levels to enhance the neuromuscular function of SOD1^{G93A} animals.

The KL-OE SOD1^{G93A} mice model was generated to overexpress KL under the EF1 α promoter (line EFmKL48) and has no detectable overexpression in muscles (Kurosu et al., 2005; Zeldich et al., 2019). However, it presents a high expression in the kidney, brain, and testis. This would explain the poor outcomes observed in their neurological scoring system and the hanging wire grip test, compared to our results. While the authors did not assess serum levels, it is reasonable to expect elevated circulating KL levels produced by the kidney, potentially explaining the increased survival rates. Considering all, we speculate that elevated KL concentrations in muscle are fundamental for neuromuscular preservation in the ALS SOD1^{G93A} mice, whereas high KL serum concentrations appear to be associated with extended life expectancy (Fig. 52).

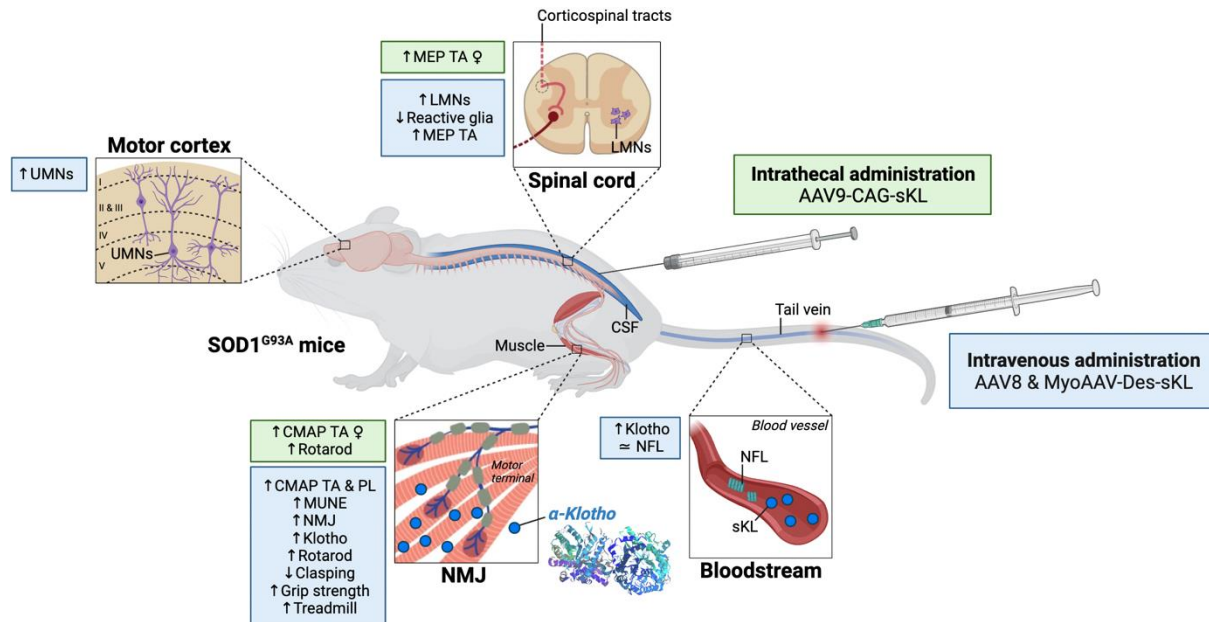


Figure 52. Therapeutic outcomes of sKL overexpression in SOD1^{G93A} transgenic mice. Intrathecal administration of AAV9-CAG-sKL and its effects on neuromuscular function and rotarod are depicted in green. Intravenous administration of AAV8- or MyoAAV-Des-sKL for muscle overexpression of sKL, and its outcomes in neuromuscular and motor function, as well as in histological and biochemical markers, are represented in blue. (Designed with Biorender, images retrieved from Taylor et al., 2016).

Molecular mechanisms underlying sKL neuroprotection in SOD1^{G93A} mice

To better understand the mode of action of sKL in SOD1^{G93A} treated animals, we performed RNAseq and proteomic analyses of the lumbar spinal cord. Despite single-cell (sc) RNAseq can provide valuable insights between cell types, especially in the context of the spinal cord, we chose bulk RNAseq over scRNAseq for its reproducibility and easier correlation and integration to the proteomic data (Gibson, 2022; Mou et al., 2020).

Females were selected for this study due to their reduced phenotypic variability compared to males and their higher responsiveness to the treatment. Secreted KL overexpression in the muscles of WT mice did not significantly alter the gene expression patterns in the spinal cord, as between these groups 51 DEGs were found but no altered proteins were detected. Samples were obtained from young mice, of 16 weeks of age, and thus indicate that sKL does not significantly alter general transcript and protein levels in youthful non-pathological conditions. When we compared treated to untreated SOD1^{G93A} mice we found 27 DEGs and no differentially represented proteins (Tables 3 and 4). In both cases, however, many transcriptionally active genes were differentially regulated but their fold change was smaller than two. Thus, small changes in the expression of these genes could collectively alter pathways or cellular processes. To further increase the significance of our omics results we performed hypergeometric tests on the top/bottom differentially expressed genes/proteins. This allowed us to assess the overlap between the two datasets and identify genes/proteins that are consistently differentially expressed across both molecular levels. Additionally, the concordance between RNAseq and proteomics serves as a biological validation between the technologies.

Some of the most enriched GO involved in both studies comprised regulation of **synaptic activity** and assembly, **neurotransmitter uptake**, or **axonal transport** (Fig. 43 and 45). This is of importance as some authors describe ALS as a synaptopathy. Synaptic dysfunctions precede MN death starting from day 21 for UMNs and from day 4 for LMNs in the SOD1^{G93A} model (M. Fogarty, 2019). Protein aggregates in ALS challenge synaptic proteostasis, especially affecting neuron synapses with poorly soluble supersaturated metastable proteins (Ciryam et al., 2015). With the overexpression of sKL we detected an upregulation in mRNA and protein levels of synaptotagmins (SYT) 2, 6, 7, and 17, as well as synaptophysin (SYN), α -synuclein (SCNA), synaptosome binding protein 91 (SNAP91) or syntaxin-binding protein 1 (STXB1).

Synaptotagmins are supersaturated proteins enriched at synapses that play a crucial role in synaptic function. Specifically, SYT-2 facilitates the synchronous **fusion of vesicles and rapid neurotransmitter release**. SYT-2-deficient mice experience severe motor dysfunction and do not

survive beyond three weeks of age. The NMJ in these mice exhibits a significant reduction in evoked release and alterations in short-term synaptic plasticity (Pang et al., 2006). SYT-7 functions by placing dense-core vesicles near membranes to facilitate UNC13 and Ca^{2+} -dependent priming, preventing vesicle accumulation beyond the reach of fusion complexes (Tawfik et al., 2021). Variants of the *UNC13A* gene have a direct link to an increased risk of ALS/FTD (A.-L. Brown et al., 2022; Ma et al., 2022). Our findings indicate a tendency for both mRNA and protein levels of UNC13A and UNC13C to be upregulated, approaching those of the wild type. On the other hand, SYT-17 orchestrates the import of vesicles from the endoplasmic reticulum to support **neurite outgrowth** and **facilitate axon regrowth** after injury (Ruhl et al., 2019). While not reaching statistical significance, *Snca* levels consistently showed a 50% increase in mRNA and protein in treated SOD1^{G93A} mice. Reduced levels of its membrane-bound state can negatively impact various steps in synaptic vesicle clustering, docking, or the homeostasis of synaptic vesicle pools (Gao et al., 2023).

Multiple **solute carrier proteins** involved in membrane and synaptic transport were also positively enriched in treated SOD1^{G93A} spinal cords compared to mock controls (Fig. 43). For instance, *Slc9a1* contributes significantly to intracellular pH homeostasis and its deletion in mice leads to neurodevelopmental disorders and early mortality (G. A. Cox et al., 1997; Iwama et al., 2018). *Slc25a24* operates as an ATP-Mg/Pi carrier, regulating the adenine nucleotide pool in the mitochondrial matrix and thereby influencing **ATP production** (Harborne et al., 2017). *Slc24a2*, on the other hand, functions as a neuronal sodium/calcium exchanger, playing a crucial role in **synaptic plasticity**. This carrier is essential for the proper functioning of axons and presynaptic terminals (Hassan & Lytton, 2020). Aligning with our results, a recent study has identified these carriers enriched in iPSC-derived LMNs and UMNs, which were downregulated upon exposure to proteotoxicity (Fiore et al., 2023).

Of note, other carriers upregulated at mRNA/protein level after sKL overexpression include *Slc30a3*, *Slc18a3*, and *Slc12a5*. *Slc30a3* is a zinc transporter mainly distributed in synaptic vesicles of glutamatergic neurons for inward zinc transport. This carrier is decreased in the spinal cord of ALS patients (Kaneko et al., 2015) and its overexpression **protects from ER stress** by ERK1/2 activation (Kurita et al., 2016). *Slc18a3* is the vesicular acetylcholine transporter (VACHT), which shows reduced immunoreactivity in SOD1^{G93A} mice at presymptomatic stages (Gallart-Palau et al., 2014; Schutz, 2005). We also noted an increase in the mRNA levels of *Slc12a5*, also known as KCC2, although this was only modest when compared to protein expression. Some authors have reported that KCC2 is downregulated in the membrane of vulnerable MNs in the SOD1^{G93A} mouse model and UMNs from post-mortem SOD1, C9ORF72, and sporadic ALS patients (Fuchs et al., 2010; Khademullah et al., 2023). Additionally, compounds that enhance KCC2 have been shown to delay the onset of motor deficits and extend the lifespan of SOD1^{G93A} mice.

While the neuronal function of KL and its involvement in synaptic activity have not been specifically investigated in the spinal cord, various studies highlight its significance for regulating brain neuronal function. For instance, KL knockout mice exhibit a reduction in the number of dopaminergic neurons, hippocampal neurons, and Purkinje cells (Kosakai et al., 2011; Kuro-O et al., 1997; Shiozaki et al., 2008). Additionally, compared to wild-type mice, KL knockout mice demonstrate signs of neuronal degeneration in the cortex and hippocampus. The brains of these mice exhibit reduced expression of synaptic proteins, along with a decrease in the number of synaptic vesicles and synapses (Li et al., 2004; Shiozaki et al., 2008). Indeed, KL has been detected in pre- and post-synaptic compartments of the hippocampus, suggesting direct physical interaction with synaptic proteins (Li et al., 2017). Moreover, overexpression of the secreted form improves cognition and memory formation by increasing synaptic transmission and enhancing activity-dependent synaptic plasticity (Li et al., 2019). Regarding cholinergic synapses, the activity of acetylcholine, choline acetyltransferase, and acetylcholinesterase are significantly impaired in KL mutant mice (Park et al., 2013). We found that in sKL-treated SOD1^{G93A} mice, the levels of ChAT and the M2 muscarinic autoreceptor were positively regulated, while the expression of cholinesterase was negatively regulated. In both cases returning to close-to-wild type levels and indicating a **restoration in the cholinergic function** of MNs.

Another component of abnormal phase separation in ALS/FTD involves the creation of stress granules (SG), whose degradation is impaired in ALS. A recent study has revealed that the development of irregular SGs, induced by mutant FUS, occurs before the loss of synaptic proteins. These abnormal SG dynamics are, in part, a result of the interaction between FUS and the RNA splicing protein muscleblind 1 (MBNL1) (Casci et al., 2019). In our screening, we detected muscleblind 2 (MBNL2), a protein with similar functions to MBNL1, as one of the most abundant proteins that were corrected by the treatment. The treatment corrected dysregulation in other proteins involved in RNA metabolism, such as SM-like protein LSM7 and HAX-1 (Fig. 45). Both are RBP proteins implicated in translation, ribosome biogenesis, rRNA processing, and SG formation (Balcerak et al., 2022; Lindström et al., 2022). In addition, treatment attenuated the overexpression of ribosomal proteins and rRNA, revealing a novel function for sKL in **addressing RNA disturbances**, a common pathological hallmark in all forms of ALS.

At the protein level, several genes involved in proteostasis were corrected by the therapy (Fig. 45). These included proteins related to **proteolysis**, such as the proteasome 10 subunit (gankyrin), adaptor SH3BGR, carboxypeptidase N, or RNF13 ubiquitin-protein ligase. For instance, gankyrin, which interacts with other subunits, plays a role in recognizing, unfolding, and translocating ubiquitinated proteins into the catalytic core of the constitutive proteasome for degradation (Whitby & Hill, 2007).

Integration of RNAseq and proteomic data further confirmed the **contribution of sKL to mitigating neuroinflammation** (Fig. 50). Notably, the expression of the classical MHC class I components H2-D1, H2-K1, and β 2-microglobulin showed a correction, with H2-D1 showing a remarkable ~10-fold decrease following the treatment. In neurodegenerative processes, aging, and axotomy, MHC-I expression is induced on resident cells, including neurons, a process mediated by INF γ . This induction is often followed by the infiltration of inflammatory cells into the affected tissue (Kellogg et al., 2023; Maehlen et al., 1988; H. Neumann et al., 1995). This phenomenon was described in the spinal cord of the SOD1^{G93A} model, where a pronounced upregulation of MHC-I expression is observed at the onset of the disease. Remarkably, these MHC-I molecules preferentially accumulate in motor axons and NMJ, suggesting that MNs produce MHC-I complexes with autoantigenic peptides and expose them to the environment. As the blood-spinal cord barrier is compromised, this may lead to a potential direct interaction with infiltrating cytotoxic T cells (Nardo et al., 2013). Reduction of KL has been shown to induce MHC-II expression through the regulation of IFN-related genes, hinting at a similar mechanism possibly occurring for MHC-I (Zhu et al., 2018).

In the spinal cord of SOD1^{G93A} mice, **sKL downregulated several inflammatory factors** (Fig. 45), including macrophage-expressed gene 1 protein, monocyte chemoattractant protein-1, FGFr, α_2 -macroglobulin, interferon-induced IFIT-family proteins, vimentin and GFAP. The downregulation of these proinflammatory factors provides a plausible explanation for the reduced activation of microglia and confirms the reduction in fibrosis and astrogliosis observed in the histological evidence.

Omics analyses revealed a significant and consistent mRNA and protein decrease of metallothioneins 1 and 2, which are specifically expressed in astrocytes, and of metallothionein 3 in neurons (Fig. 45B). Metallothioneins function as scavengers of toxic heavy metal ions and free radicals. Alterations in metallothionein expression precede the onset of motor deficits in SOD1^{G93A} mice and sALS patients (Gong & Elliott, 2000; Hozumi et al., 2008). The hypothesis suggests that metallothioneins may serve as early and crucial protective factors in ALS. Thus, the reduction in the activation of these proteins indicates that **sKL is mitigating metal and oxidative toxicities**.

Concerning this, comparison of mRNA and protein pathway enrichment analyses revealed consistent enrichment in **mitochondrion organization, mitochondrion transport, and mitochondrial RNA metabolic process** GOs in sKL- compared to Null-SOD1^{G93A} mice (Fig. 50). Expression of proteins involved in the normal function of the respiratory chain and protein transport mechanisms was corrected at the mRNA/protein level. This includes ATP synthase, complex I and cytochrome c oxidase components, or the Timm22 and Timm44 translocases. Proteins involved in **mitochondrial protein synthesis** and quality control were also enriched. Taken together, these

findings imply a revitalized functionality in **mitochondrial respiration, ATP production, membrane maintenance**, and protein regulation within these cellular organelles affected in ALS (Liu et al., 2004; Wiedemann et al., 2002)

Concerning **apoptotic alterations**, we observed consistent dysregulations in AIMF1, HAX-1, annexin V, and Bcl-2-like proteins (Bcl-xL and Bcl-xS). AIMF1 functions as a proapoptotic protein, playing a role in folding the subunits of the mitochondrial respiratory chain. In conditions such as ALS, AIMF1 is released from mitochondria and translocates to the nucleus promoting chromatin condensation and DNA fragmentation (Wischhof et al., 2022). Conversely, HAX-1 acts as a mitochondrial antiapoptotic protein, exerting neuroprotective effects against neuronal injuries (Fadeel & Grzybowska, 2009; Sui et al., 2021). Alterations in KL levels have been reported to induce changes in apoptotic markers. KL knockout mice exhibit downregulation of anti-death proteins Bcl-2 and Bcl-xL, and upregulation of the proapoptotic factor Bax (Nagai et al., 2003; Shiozaki et al., 2008). KL supplementation in cell cultures has the opposite effect on these genes and reduces the number of annexin V-positive apoptotic cells (Hu et al., 2021; Yamamoto et al., 2005).

Molecular mechanisms underlying sKL neuromuscular preservation in SOD1^{G93A} mice

Since our therapy targets sKL to muscles, we analyzed gastrocnemius muscles at the transcriptomic and proteomic levels. Skeletal muscle is as a major contributor to ALS pathology, with molecular abnormalities leading to NMJ disassembly and axonopathy, undergoing physiological changes before denervation occurs (Tsitkanou et al., 2016; Wong & Martin, 2010). The same female mice as in the spinal cord analyses were used in this study. Similarly, overexpression of sKL slightly altered mRNA gene expression patterns, by differentially regulating 44 genes, but we did not detect any distinctively represented protein. Thus, suggesting that sKL overexpression has no noticeable effects in young muscles. In contrast, sKL had a significant impact on SOD1^{G93A} muscles, with 119 DEGs and 180 altered proteins (Tables 5 and 6). The treatment especially upregulated proteins, suggesting mostly a correction of those endogenously downregulated compared to wild-type animals.

Functional comparison between RNA and protein detected that most upregulated pathways in SOD1^{G93A} sKL compared to SOD1^{G93A} null were related to **muscle contraction and development**. Of these, some of the most upregulated proteins in SOD1^{G93A} muscles were Ankrd1, Col19a1, and Rrad, which we found corrected at mRNA and protein levels by the treatment (Fig. 47 and 49B). Calvo and collaborators (2012) identified the mRNA of these genes as good correlators of disease

progression and significantly altered compared to the early symptomatic stage. Other genes the authors described, and that we also found dysregulated at the mRNA level include *Myod1*, *Myog*, *Sln*, *Mt2*, and *Snx10*. However, we failed to detect their protein levels by MS/MS. Ankyrin repeat domain-containing protein 1 (*Ankrd1*) plays a crucial role in the plasticity of skeletal muscles and seems to serve as a universal indicator of muscle damage when elevated (Laure et al., 2009).

Conversely, regenerative mechanisms typically counteract the imbalance in SOD1^{G93A} muscles by either maintaining tissue integrity and supporting skeletal myogenesis, as is the case of collagen type XIX α 1 (*Col19a1*) (Sumiyoshi et al., 2004), or by promoting muscle differentiation and regeneration, as is the case of myogenic differentiation 1 (*Myod1*) and myogenin (*Myog*) (Cao et al., 2006; Manzano et al., 2011). Expression levels of *Ankrd1*, *Col19a1*, *Sln*, *Myod1*, *Myog*, and *Snx10* correlated significantly and inversely with SOD1^{G93A} mice longevity (Calvo et al., 2012, 2019). Thus, indicating that the correction observed in the expression of these genes could improve ALS prognosis. Noteworthy, we successfully induced a decrease in the expression of the RBP Zfp697. Zfp697 was recently identified as a damage-induced regulator of muscle regeneration, whose sustained upregulation results in gene expression patterns associated with chemokine secretion, immune cell recruitment, and extracellular matrix remodeling during muscle regeneration (Correia et al., 2023).

Elevated levels of sKL within the muscle corrected various **collagen and structural genes associated with fibrosis and atrophy** (Fig. 47E and 49B). These included numerous collagen proteins, MFAP41, IGFN1, activin, and TGF- β . Notably, KL is recognized as an inhibitor of the TGF- β /Wnt axis and mitigates fibrosis and sarcopenia in renal and muscle tissues (Doi et al., 2011; Ohsawa et al., 2023; Yousef et al., 2015; Q. Yuan et al., 2022). We also noted a trend toward the downregulation of downstream targets in this pathway, including multiple SMAD proteins and FRZB, both identified as muscle biomarkers in ALS (Kwan et al., 2020; Si et al., 2014). Interestingly, DKK1, an alternative Wnt pathway inhibitor, exhibited strong upregulation in SOD1^{G93A} mice, likely as an effort to counter fibrosis and induce myogenesis (Ahrens et al., 2018b). sKL overexpression rectified its expression, suggesting that sKL alone was effective **in inhibiting fibrosis**, eliminating the need for additional Wnt inhibition.

sKL upregulated the expression of nicotinamide nucleotide transhydrogenase (NNT), which transfers electrons from NADH to NADP⁺. This is important as the glutathione (GSH) and Trx systems rely on the reducing power of NADPH to effectively combat **oxidative stress** (N. Xie et al., 2020). While levels of proteins involved in GSH synthesis remained unchanged by sKL overexpression, we detected an increase in cysteine metabolic processes (Fig. 51B), a direct precursor of glutathione. Several enzymes of the glutathione S-transferases family, which are involved in cellular detoxification of electrophilic compounds, were upregulated by the treatment.

We also found a significantly enhanced expression of NAD(P)H dehydrogenase (NQO1), Thioredoxin-related transmembrane protein 4, thioredoxin-domain containing proteins, and the ER-resident thioredoxin-like Selenof (Fig. 47I), indicating a possible **activation of Nrf2 antioxidant signaling**. Impairment of the Nrf2 system has been described in ALS, as a mechanism to counteract oxidative stress in the spinal cord and muscles (Kraft et al., 2007; Mimoto et al., 2012). Despite the deletion of Nrf2 has no impact on the disease course of SOD1^{G93A} mice (Guo et al., 2013; Vargas et al., 2013), Nrf2 activators or its selective overexpression in astrocytes shows neuroprotective effects (Neymotin et al., 2011; Vargas et al., 2008). Thus, we can infer an activation of Nrf2 by sKL in our approach, as previously described in other disease models (Maltese et al., 2017; Xing et al., 2021; Zhao et al., 2022)

Oxidative stress can induce high aberrant expression of RRAD as described in previous studies with SOD1^{G86R} mice (Halter et al., 2010), leading to an alteration in muscle excitation-contraction coupling. This alteration in muscle function may be further influenced by increased expression of sarcolipin (SLN), as SLN triggers cytosolic calcium influx and plays a regulatory role in relaxation-contraction cycles (Vasu et al., 2009). SLN binds to and interacts with SERCA2 isoforms to trigger the transition of skeletal muscle from glycolytic to oxidative metabolism. A previous study has shown that overexpression of SLN precedes the upregulation of denervation markers and orchestrates metabolic reprogramming and bioenergetic defects. Indeed, inhibition of fatty acid oxidation led to improved motor function in SOD1^{G93A} mice (Scaricamazza et al., 2020). Additionally, in connection with calcium homeostasis, higher levels of Calsequestrin 1 and 2 (CSQ1,2) were detected in sKL SOD1^{G93A} muscles, which are the main **Ca²⁺-buffering proteins** within the sarcoplasmic reticulum (Woo et al., 2020).

The **inflammatory state** induced by ALS was reduced in the muscles of SOD1^{G93A} mice overexpressing sKL (Fig. 49A and 51A). The MHC class I receptor H2-D1 was one of the major significantly upregulated proteins, also in the spinal cord. As previously mentioned, H2-D1 is present in the soma of MNs but is predominantly expressed presynaptically at the NMJ within the muscles (Nardo et al., 2013). Following peripheral nerve injury, there is a notable increase in H2-D/K at the cut ends and growth cones of MNs (Thams et al., 2009). Elevated MHC-I levels suggest an effort for denervated axons to regenerate, but they are also indicative of increased denervation in untreated animals compared to SOD1^{G93A} sKL mice.

In addition, we found that tapasin was increased in both, muscles and the spinal cord, which plays a role in autopeptide loading and the stable assembly of MHC-I (Jiang et al., 2022). Cytotoxic CD8⁺ T cells have been found in close contact with the NMJ, suggesting that they play a role in degrading defective terminals or clearing debris (Chiu et al., 2008). Nevertheless, they could be a double-edged sword as they secrete proinflammatory substances and trigger a cytotoxic immune

response that also leads to direct damage of motor axons and initiates a cascade leading to MN death in SOD1^{G93A} mice. This could be exacerbated by infiltration of activated macrophages in the muscles, as we found a remarkable upregulation of the Iba1 marker in Null SOD1^{G93A} muscles. Although this was not completely reversed by the treatment, we identified a significant 3-fold correction in the levels of this marker, which we further need to confirm by immunohistology. A similar decrease was observed for the integrin $\alpha 5$, identified in a recent study in microglia around motor corticospinal tracts and peripheral nerve macrophages with distinct proinflammatory properties in late-stage SOD1^{G93A} mice and ALS patients (Chiot et al., 2023). The **reduced recruitment of inflammatory cells** in the muscles of treated SOD1^{G93A} mice might be attributed, among others, to the diminished release of MIF, MIF-2, galectin-3, or Emilin1 (Fig. 49B and 51A), proinflammatory factors known for sustaining inflammation (Calandra et al., 2003; F.-T. Liu & Hsu, 2007; Merk et al., 2012; Pivetta et al., 2022).

DNA topological changes emerged as one of the significant GO annotations in the transcriptomic and proteomic comparison. Especially, expression of topoisomerases 1 and 3 β was diminished and corrected to wild-type levels (Fig. 49B). Although its exact contribution in ALS has not been reported, topoisomerase-induced DNA damage can be promoted by different stressors and is beginning to be recognized as frequent in neurological disorders (Crewe & Madabhushi, 2021).

Another enriched mechanism when comparing sKL- to null-treated SOD1^{G93A} muscles was **ubiquitin-mediated proteolysis** (Fig. 47A). Protein aggregation of SOD1, RBPs, and other misfolded proteins is an important hallmark of the SOD1^{G93A} mouse model. Primary degradation pathways to eliminate unwanted or misfolded proteins –the ubiquitin-proteasome system and the autophagy-lysosomal pathway– depend on the modification of targets by ubiquitin (Schmidt et al., 2021). We uncovered a novel function of sKL in upregulating numerous ubiquitin-protein ligases, E3 ubiquitin-protein ligases, ubiquitin conjugation factors, and ubiquitin-conjugating enzymes, thus **maintaining proteostasis**. On the contrary, the deubiquitinase UCH-L1 was significantly downregulated, which has been shown to promote aggresome-like inclusions in response to proteasome impairment in neurodegenerative diseases (Ardley et al., 2004). Furthermore, UCH is mainly expressed in slow twitch muscle fibers, and its depletion was associated with muscle growth and enlarged muscle fiber size by inhibiting the mTORC1 activity (H. Gao et al., 2019). Protein levels of sequestosome 1 (p62) were partially corrected by the treatment. Protein p62 co-depositions with SOD1 and other aggregation-prone proteins in ALS, further inducing their aggregation (Gal et al., 2007; Trist et al., 2022).

This enhanced proteolysis could explain why other **aggregation-prone proteins displayed a remarkable reduction**, including EWSR1, FUS, and hRNP2B1. Mutations in these RBPs have been identified as causative for ALS (Couthouis et al., 2012; Hewitt et al., 2010; Kim et al., 2013;

Vance et al., 2009). TIAL1 was also downregulated by the treatment and, as its paralog TIA-1, through its prion-like domains probably forms cytoplasmic inclusions being involved in stress granule formation (D.-Y. Lee et al., 2020; Sekiyama et al., 2022). Significantly, exportin-5 exhibited a 48-fold decrease with sKL upregulation (Fig. 49B), likely indicating a mechanism to prevent excessive RNA export and mislocalization to the cytoplasm and aberrant miRNA biogenesis (Chen et al., 2022; Kim et al., 2014). Many additional proteins associated with RNA binding, processing, and transport, together with their corresponding mRNA levels, were reduced to a similar degree of wild-type or near-wild-type conditions.

Proteins associated with **axogenesis and axonal pathfinding** exhibited increased abundance in the muscles of SOD1^{G93A} animals overexpressing sKL. For instance, the triple functional domain protein (TRIO) showed downregulation in null mice, and its levels were effectively corrected by sKL. TRIO, implicated in axon outgrowth and guidance, colocalizes with Kiddins 20 at neurite tips and is linked to downstream regulation of growth factors (Neubrand et al., 2010; van Haren et al., 2014). Additionally, neurofilament proteins, crucial for axonal regeneration, maintaining axon caliber, and facilitating electrical impulse transmission (Yuan et al., 2012), exhibited substantial upregulation of their mRNA levels in response to sKL. This led to the normalization of protein levels to those observed in wild-type conditions, suggesting **preservation of axons and neurofilament proteins within muscles** (Fig. 49A), even in the absence of notable changes in NFL serum concentrations (Fig. 33J).

Finally, sKL enriched genes and proteins belonging to the **mitochondrial organization and MAM** gene ontology terms in SOD1^{G93A} muscles. Similarly to the spinal cord, most upregulated proteins fall in functions related to mitochondrial metabolism, such as GPDH, COX20, ALDH1L2, HIG1, dicarboxylate carrier, or glutaminase. Proteins involved in **balancing the redox state** showed increased abundance in the muscles of treated animals, with a notably high expression observed for NNT (N. Xie et al., 2020). Importantly, the levels of lipoyl synthase were effectively restored by sKL treatment. This synthase plays a crucial role in lipoic acid synthesis –a potent thiol antioxidant– that serves as a cofactor for various mitochondrial enzymes (Hendricks et al., 2021). As for proteins enriched within the MAM regions, we identified TOMM20, responsible for translocating nuclear-encoded proteins, or TMX2, which harbors a thioredoxin-like domain and regulates redox balance, protein folding, and transport (Franco-Iborra et al., 2018; Oguro & Imaoka, 2019; Vandervore et al., 2019). Irregularities in these proteins and disruptions in mitochondrial protein import are recognized factors that can lead to mitochondrial dysfunction.

On the other hand, expression of the calcium uniporter protein (MCU), calcium uptake proteins (MICU), and mitochondrial glutamate carrier 1 (Slc25a22) were downregulated by sKL. These transporters are probably upregulated in SOD1^{G93A} mice to compensate for increased calcium

concentrations and to promote mitochondrial buffering capacity but ultimately induce mPTP opening and apoptosis (Tadic et al., 2014). Thus, inducing the expression and activation of Bax and caspases and p21-mediated cell senesce, all of which were partially reversed by the treatment. The effects of sKL on **avoiding mitochondrial dysfunction** in the muscle are thought to mainly rely on counteracting ROS production, as KL depletion results in cardiolipin lipid and cytochrome c oxidation, leading to the loss of cristae structure (Sahu et al., 2018). Moreover, KL protection from apoptosis and senesce has been reported in several *in vitro* and animal models (Ikushima et al., 2006; Maekawa et al., 2011; Shaker et al., 2021; Sugiura et al., 2010).

Overall, the analysis of the transcriptomic and proteomic data allowed us to confirm the multiple beneficial effects of secreted KL on the altered pathological mechanisms in ALS. It illustrates that although the most substantial corrections occur in the muscle, KL secretion can also influence the spinal cord by subtly altering its expression patterns (Fig. 53). This research has even uncovered new roles for KL, including its involvement in **proteostasis** and maintenance of **RNA homeostasis**.

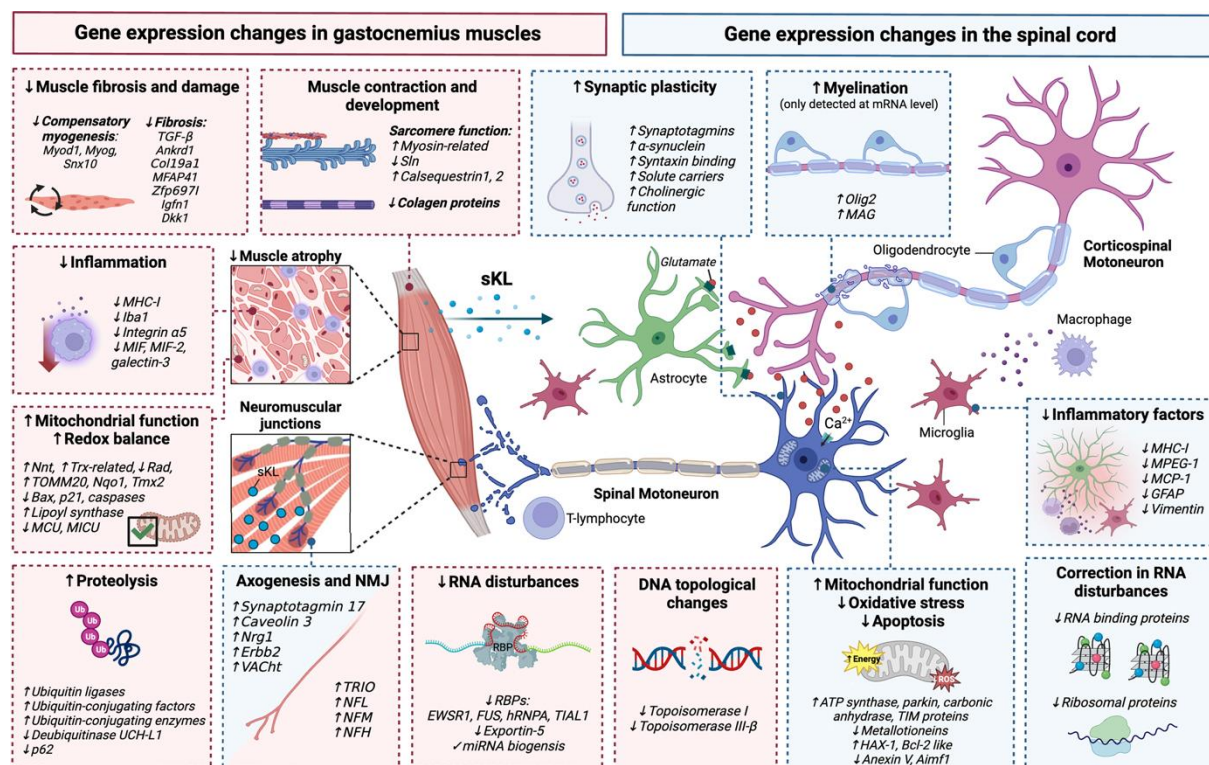


Figure 53. Changes in gene expression patterns induced by muscle secretion of sKL in SOD1^{G93A} mice. Each panel illustrates biological processes and genes differentially regulated in sKL-treated gastrocnemius muscles (light red) or spinal cords (light blue). Secreted KL induced similar pathways in the gastrocnemius muscle and spinal cord, however, key genes driving these mechanisms differ in each tissue. (Designed with Biorender, NMJ image extracted from Taylor et al., 2016)

Future perspectives and translational potential of sKL gene therapy for ALS

In this thesis, we have demonstrated that targeting sKL overexpression is a viable therapeutic approach to preserve neuromuscular function, enhance motor performance and strength, preserve muscle mass, reduce neuroinflammation, and prolong the survival of SOD1^{G93A} mice. Additionally, this intervention has been effective in mitigating multiple underlying pathological mechanisms in ALS. Despite muscle overexpression of sKL appears to hold more promise than CNS overexpression, we are considering future studies that will involve a combination of intravenous and intrathecal or intracortical administrations. We believe that a combined therapeutic approach could strengthen the protective mechanisms identified in the spinal cord, potentially leading to synergistic effects for both spinal and cortical MNs, which have been difficult to obtain so far (Mòdol-Caballero, et al., 2021b).

Modification of the viral capsid and the reduction in the dose for our intravenous therapy targeting the muscles were driven in part by considerations of translational potential and feasibility in patients' use. Since bringing gene therapies to the clinic is challenging (Kohn et al., 2023), evaluating the therapeutic administration of recombinant sKL protein or mRNA is also a viable option. However, alternative therapeutic carriers such as liposomes or nanoparticles need to be explored (Wang et al., 2023), as an increase in systemic sKL was insufficient in preserving neuromuscular function in our preclinical studies. To improve the expression of sKL in muscles and refine our therapeutic approach, the next efforts may focus on increasing sKL levels through increased expression and secretion rather than further escalating AAV doses. Given that the observed fold-change in sKL expression is lower than expected at the tested dose, we suspect that sKL mRNA might undergo the NMD pathway, resulting in a majority of mRNA molecules not being translated into protein. To substantiate this hypothesis, it may be of interest to conduct mRNA expression analysis and assess the number of viral genomes per cell. Further to integrating the WPRE sequence into our construct, we are exploring other strategies to enhance sKL expression. Codon optimization is envisioned as a crucial step, to address issues related to the usage of rare codons, adapt to tRNA abundance, enhance mRNA stability, and mitigate secondary structures that hinder efficient translation (Mauro & Chappell, 2014). Moreover, the incorporation of a Kozak sequence or internal ribosome entry sites (IRES) could facilitate protein translation (Acevedo et al., 2018; Renaud-Gabardos, 2015). Subsequent steps will also involve *in vitro* testing of novel synthetic muscle-specific promoters, including the chimeric MCHK7 or the SPc5-12 promoters, that are possibly stronger than the desmin promoter (Skopenkova et al., 2021). In essence, a comprehensive analysis of our construct will be conducted to determine the most appropriate modifications for enhancing sKL expression in muscle cells.

For ensuring the path to clinical translation, the strategy developed in this thesis, i.e. overexpression of sKL through production in skeletal muscles to provide widespread contact with motor units, should be also assayed in other models of MN degeneration, either of genetic cause or of traumatic origin.

CONCLUSIONS

Chapter I: Validation of Klotho in models of motoneuron degeneration. Therapeutic overexpression of Klotho in the spinal cord of SOD1^{G93A} mice.

1. KL mRNA expression is markedly reduced in the SOD1^{G93A} ALS mouse model at the end stage, with transcripts exhibiting distinct tissue regulation.
2. mRNA expression of KL transcripts is markedly reduced in the forelimb muscles of the BPI model and correlates with atrophy. Secreted KL transcripts decrease significantly in the spinal cord following BPI.
3. KL isoforms provided by AAV vectors promote MN survival under acute excitotoxicity in SCOCs.
4. Intrathecal administration of AAV9-CAG-sKL partially preserved neuromuscular function and locomotor performance in female SOD1^{G93A} mice.

Chapter II: Targeting skeletal muscle-specific overexpression of Klotho with systemic AAV8 vectors.

1. Overexpression of sKL in skeletal muscle by i.v. administration of AAV8-Des-sKL at a dose of 3×10^{14} vg/kg, but not at 1.8×10^{14} vg/kg promotes significant preservation of neuromuscular function, and motor performance, resulting in a delayed disease onset of female SOD1^{G93A} mice.
2. Secretion of KL from the transduced muscles protects spinal MN, reduces microglial and astrocyte reactivity in the spinal cord, and preserves NMJ innervation in the muscles.

Chapter III: Enhanced Klotho delivery to muscles with the novel myotropic MyoAAV vector.

1. MyoAAV-mediated overexpression of sKL in skeletal muscle of SOD1^{G93A} mice shows a dose-response improvement in neuromuscular function. The highest dose, still ~19 times lower than for AAV8, preserves the number and size of MU, motor function, and strength, without causing adverse effects. Viral-mediated therapy with sKL prolongs the survival and the disease onset of male and female SOD1^{G93A} mice.
2. Muscle-secreted KL protects the number of spinal MNs, reduces neuroglial reactivity, and preserves NMJ innervation.
3. Treatment given at a symptomatic stage of SOD1^{G93A} mice with sKL preserves remaining MNs and NMJ, resulting in a slowed decline in neuromuscular and locomotor functions.

4. Overexpression of sKL in skeletal muscles is essential to preserve neuromuscular function in SOD1^{G93A} mice, as a systemic increase in sKL expression and serum levels prove insufficient.

Chapter IV: Transcriptomic and proteomic studies unravel Klotho mechanisms of protection in ALS.

1. Muscle secretion of sKL leads to changes in gene and protein expression patterns in the spinal cord of SOD1^{G93A} mice. RNA sequencing and proteomic studies identified corrections of several altered pathways, highlighting synaptic function and axogenesis, RNA perturbations, proteostasis, neuroinflammation, oxidative stress, mitochondrial dysfunction, and apoptosis.
2. Overexpression of sKL also corrects gene and protein expression patterns in the skeletal muscles of SOD1^{G93A} mice, including muscle integrity and development, fibrosis, proteolysis, oxidative stress, inflammation, DNA topological changes, RNA alterations, axogenesis, mitochondrial dysfunction, and apoptosis.
3. Functional omics studies confirmed the pleiotropic nature of KL, validated its efficiency in ALS treatment, and uncovered new roles in protein and RNA homeostasis.

MATERIALS AND METHODS

1. DNA cloning techniques

1.1. Therapeutic construct and plasmid generation

Various plasmids were employed or created to generate gene therapy vectors. Plasmids essential for viral vector production (helper plasmid pXX6 and pRep2CapX), as well as those bearing the foundational structure of gene expression cassettes (backbones) encompassing promoter and polyadenylation (polyA) sequences, were acquired from the Unitat de Producció de Vectors (viralvector.eu) at the Universitat Autònoma de Barcelona. Constructs containing the genes of interest or GFP originated from past research projects within our group and from external collaborators.

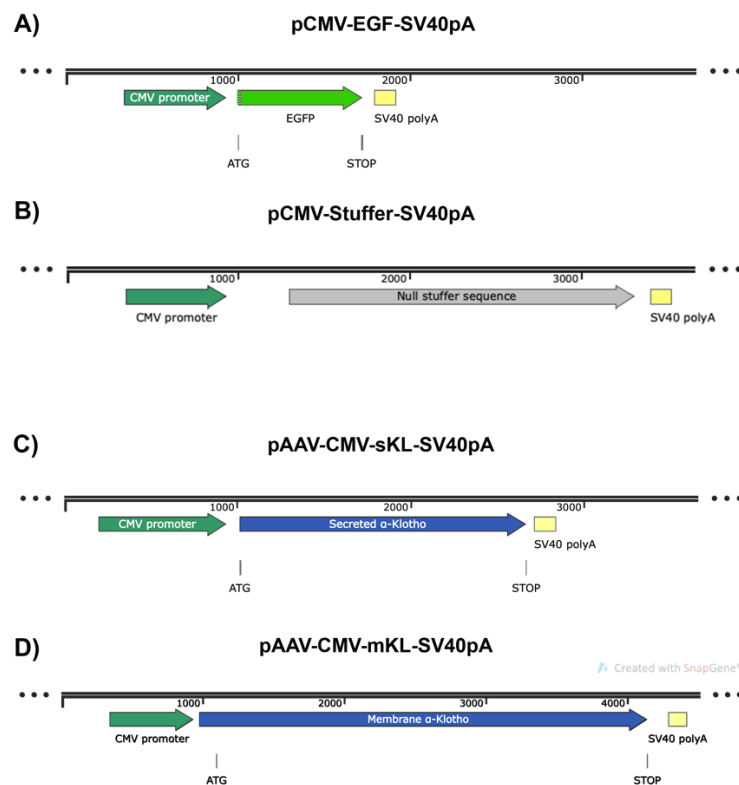


Figure 54. Plasmids with constructs used in viral vectors for *in vitro* studies. (A, B) Constructs used in Ad5 and AAV9 transduction studies in SCOCs. (C, D) Therapeutic cassettes used in AAV9 vectors for assessing MN survival in SCOCs exposed to glutamate.

The pAAV-CMV-sKL, pAAV-CMV-mKL, and pAAV-Null-mKL constructs (Fig. 54) used for *in vitro* studies were already generated in the laboratory (Massó et al., 2018; Roig - Soriano et al., 2022). The mouse KL sequences were generated by PCR amplification of the two KL transcripts cDNA, upon RNA extraction and retrotranscription. For the generation of the pAAV-CAG-sKL-mP1 construct (Fig. 55A, B), the sKL cDNA was cloned into *HpaI* and *Sall* sites to the plasmid pAAV-CAG-polylinker. This plasmid contained the promoter, the first exon, and the first intron of the chicken β -actin, with the cytomegalovirus (CMV) early enhancer and the splice acceptor of the rabbit β -globin gene (Hitoshi et al., 1991).

A segment of the mouse protamine (mP1) sequence, which contained an intron and a polyadenylation (polyA) site (Brenner et al., 1994), was included at the 3' end of sKL in the pAAV-CAG-sKL plasmid using the *NotI* site. To generate the null control construct, a non-coding stuffer sequence was cloned into the pAAV-CAG-polylinker using *BamHI* sites. This sequence corresponds to the non-coding RNA transcript variant IV of the TMEM185A gene.

For generating the pAAV-hDes-sKL-WPRE-SV40pA plasmid (Fig. 55C, D), the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was excised from a previous plasmid using *NotI* and *HincII* sites. The WPRE sequence was then incorporated into the pAAV-CAG-sKL plasmid using *NotI* and *BstZ17I* digestions. The sKL-WPRE sequence was finally cloned with the *BamHI* and *HpaI* restriction enzymes into the pAAV-hDes-polylinker-SV40pA, previously digested with *BamHI* and *BstZ17I*. The polyA signal is an essential element of the expression cassette to enhance mRNA stability and augment translation efficiency (Proudfoot, 1991). The SV40 polyA signal, derived from the simian virus 40, is among the most frequently used polyA sequences (van den Hoff et al., 1993). The WPRE is a powerful cis-acting regulatory module that enhances stability and expression by creating a tertiary structure (Kantor et al., 2014).

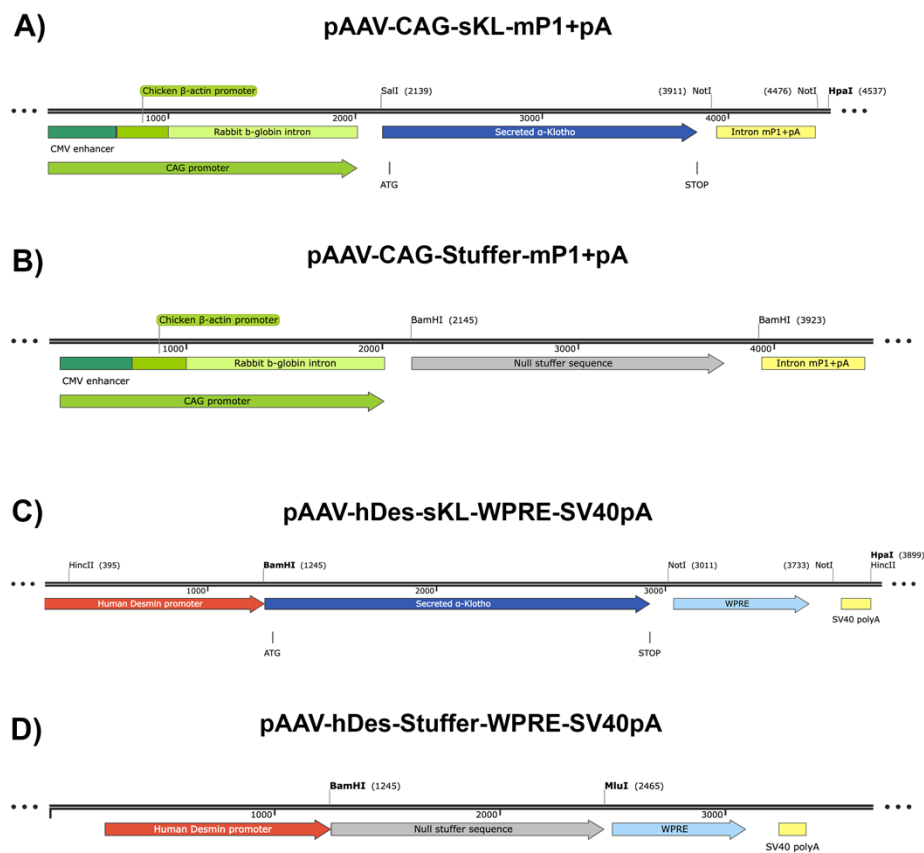


Figure 55. Plasmids with therapeutic constructs used in gene therapy studies. (A, B) Expression cassettes encapsidated in AAV9 vectors for intrathecal delivery. (C, D) Therapeutic constructs used with AAV8 and MyoAAV vectors to target specific expression to muscles following systemic administration.

The human desmin promoter is specific for striated muscle and was kindly provided by B. Byrne (University of Florida, FL, USA). The human desmin sequence spans 354 bp and incorporates a myocyte-specific enhancer factor 2 (MEF2), a MyoD enhancer element, and a TATA box to enhance transcription specificity (Pacak et al., 2008). The non-coding stuffer sequence was cloned into the pAAV-hDes-polylinker-SV40pA using *Bam*HI and *Mlu*I sites to generate the Null construct with the hDesmin promoter. These plasmids underwent amplification and cloning processes to facilitate the production of the intended gene therapy viral vectors.

1.2. Enzymatic restriction of plasmids or DNA products

For the incorporation of the genes of interest into the expression cassette backbone, plasmids underwent targeted digestion at specific sequences using various restriction enzymes (New England Biolabs or Fermentas, ThermoFisher). Reaction mixtures for digestion were prepared by combining the plasmid solution with the suitable commercial digestion buffer, 2-10 enzymatic units per μ g of DNA, and H₂O to a total volume of 20 μ L. The digestion mixture was then incubated at the temperature specified by the manufacturer for a minimum of 2 hours for complete digestion.

For preparative cloning digestion, 3 μ g of plasmid DNA was used, whereas 1 μ g was used for diagnostic digests of final plasmids. Several reaction times were employed for partial digestions, targeting desired recognition sites but potentially omitting those that are not of interest. This strategy ensures that not all possible cleavage sites are cut during the process. For sequential digestion involving incompatible enzymes, stepwise reactions were conducted considering increasing salt concentrations in enzyme buffers.

1.3. Separation and purification of digested DNA fragments

DNA enzymatic digested products were mixed in loading buffer and loaded, together with a 1Kb DNA ladder (ThermoFisher), in a 1% agarose (Amresco) gel prepared in TAE 1x buffer containing RedSafe (1:20.000, iNtRON). The gel was run in an electrophoresis cuvette containing TAE 1x buffer for 60-90 min at an electric current of 60-120V. DNA fragments within the gel were visualized in a UV transilluminator (Syngene) and the bands corresponding to the desired fragments were sliced with a scalpel. The isolated bands were purified using the Wizard® SV gel Clean-up system (Promega) following the manufacturer's instructions and eluted in 20 μ L of dH₂O.

TAE 1x: 40mM Tris-Ac pH 8.0

Loading buffer 10x: 50% Glycerol (Panreac), 100 mM EDTA pH 8.0 (USB), 1% SDS (USB), 0.1% Bromophenol blue (Sigma), 0.1% Xilencianol (Sigma).

1.4. DNA dephosphorylation and ligation

The DNA fragment corresponding to the backbone of the future plasmid (with the ampicillin resistance gene), underwent dephosphorylation to prevent DNA recircularization of the cut ends without the insertion of the desired insert. This process involved the addition of 1 μ L of FastAP™ alkaline phosphatase to 200 ng of purified backbone solution in 10X FastAP™ Buffer (Fermentas, ThermoFisher). The mixture was then incubated for 1 hour at 37°C, followed by enzyme inactivation through a 5-minute incubation at 75°C.

After dephosphorylation of the backbone, various ligation mixtures were created. These reactions included 0.25 μ L of T4 DNA ligase (New England Biolabs), 1.5 μ L of the ligation buffer, 50 ng of the plasmid backbone, molar proportions of the insert, and H₂O up to 15 μ L. The ligation reactions were of 1:1 and 1:3 molar proportions of the plasmid backbone and insert fragments. Controls for ligation were added, consisting of a 1:0 ligation reaction for assessing backbone recircularization and a reaction without ligase for assessing the presence of original plasmids that were not digested. For cohesive ends, mixtures were incubated at 16°C overnight, and for blunt ends at 37°C. T4 ligase was heat-inactivated at 65°C for 10 min. The resulting solutions were cooled and transformed into chemically competent *Escherichia coli* TOP10 cells.

1.5. Competent *E.coli* TOP10 preparation

Five microliters of chemically competent *E. coli* TOP10 (ThermoFisher) were cultured in 3 mL of LB media overnight at 37°C. The resulting cell preculture was employed to inoculate a 200 mL LB culture medium at 37°C until reaching an OD₆₀₀ of 0.5, indicating the cells were in the exponential growth phase. Subsequently, the cells were centrifuged in four 50 mL tubes for 5 min at 4,000g at 4°C. The resulting pellets were resuspended in 30 mL of TFB1 buffer per 100 mL of the initial culture and incubated on ice for 90 min. Following this, the suspension was centrifuged at 4,000g at 4°C for 5 min. The new pellets obtained were then resuspended in 4 mL of TFB2 buffer for every 100 mL of the initial culture. The cell resuspension was aliquoted at 100 μ L and stored at -80°C for future use in the transformation of competent *E. coli* TOP10 cells.

Luria Broth (LB): 15g/L of Miller's Luria Broth (Conda)

TFB1 (4°C): 100 mM RbCl (Sigma), 50 mM MgCl₂ (Panreac), 30 mM AcK (Sigma), 10 mM CaCl₂ (Panreac), 15% glycerol (Sigma), p=5.8. Sterilized by filtration with 0.22 filters.

TFB2 (4°C): 10 mM MOPS (Fluka), 10 mM RbCl (Sigma), 75 mM CaCl₂ (Panreac), 15% glycerol (Sigma), pH = 6.8. Sterilized by filtration with 0.22 filters.

1.6. Bacterial transformation and plasmid amplification

Plasmids were amplified using chemically competent *E. coli* cells (TOP10) through the heat shock procedure. This involved adding up to 10 µL of the plasmid solution to 100 µL of a competent cell suspension, followed by a 5-min incubation on ice to ensure mixture homogenization. Subsequently, cells underwent a 90-second incubation in a 42°C water bath and were then transferred back to ice for 2 min. The temperature variations facilitated the penetration of plasmids into the cells. The plasmids of interest carried ampicillin resistance genes, enabling the positive selection of properly transformed bacteria.

To induce antibiotic resistance expression, 900 µL of LB media were added, and the cells were incubated for 1 hour at 37°C in an orbital shaker incubator (Thermo Electron Corporation). Cells were pelleted by centrifugation for 1 minute at 12,000g, and the pellet was resuspended with 100 µL of LB media. Finally, the transformed bacteria were seeded on an LB-agar plate containing the selection antibiotic (ampicillin) and incubated overnight at 37°C in an incubator (JP Selecta). Bacteria carrying a version of the plasmid with antibiotic resistance proliferated and formed visible colonies.

For selection of the bacterial colonies carrying the correct plasmid, several bacterial colonies were picked and independently transferred into tubes containing 2 mL of LB media with 100 µg/mL of ampicillin. The number of colonies picked depended on the number of colonies in the ligation control LB-agar dishes. The tubes were then incubated overnight at 37°C in an orbital shaker incubator. To extract plasmid DNA, 1.5 mL of the bacterial suspension was pelleted by centrifugation for 1 minute at 12,000 g, and the cells were resuspended with 200 µL of the resuspension buffer from the E.Z.N.A.® Plasmid DNA Maxi Kit. The manufacturer's instructions were followed for the extraction of plasmid DNA.

LB-agar: 25g of LB (Conda) and 15g of Agar (Panreac) in 1L of dH₂O. The solution is autoclaved, 100 µg/mL of ampicillin (USB) are added and poured into petri plates (Nunc).

2. Viral vectors

For the transduction of SCOCs and the gene therapy experiments, several viral preparations were used or produced containing GFP, sKL, mKL, and Null sequences (Table 7). Adenovirus vectors were obtained from the Unitat de Producció de Vectors (UPV). The AAV vectors were generated during this thesis at the biosafety facilities of the UPV at the Universitat Autònoma de Barcelona.

Table 7. Viral vectors with their respective titers used in SCOCs and *in vivo* experiments

Vector and serotype	Promoter	Gene of interest	Titer
Adenovirus 5	CMV	GFP	1.50x10 ¹¹ IU/mL
Adenovirus 5	CMV	Null	1.25x10 ¹¹ IU/mL
AAV9	CMV	GFP	1.34x10 ¹³ vg/mL
AAV9	CMV	sKL	1.67x10 ¹³ vg/mL
AAV9	CMV	sKL	8x10 ¹² vg/mL
AAV9	CMV	mKL	2.06x10 ¹³ vg/mL
AAV9	CMV	Null	1.17x10 ¹³ vg/mL
AAV9	CAG	Null	1.17x10 ¹³ vg/mL
AAV9	CAG	Null	2.18x10 ¹³ vg/mL
AAV9	CAG	sKL	1.27x10 ¹³ vg/mL
AAV9	CAG	sKL	2.11x10 ¹³ vg/mL
AAV9	CAG	sKL	1.63x10 ¹³ vg/mL
AAV8	hDes	sKL	2.14x10 ¹³ vg/mL
AAV8	hDes	sKL	1.48x10 ¹³ vg/mL
AAV8*	hDes	sKL	1.97x10 ¹³ vg/mL
AAV8*	hDes	sKL	2.9x10 ¹³ vg/mL
AAV8	hDes	sKL	2.21x10 ¹³ vg/mL
AAV8	hDes	sKL	1.29x10 ¹³ vg/mL
AAV8	hDes	sKL	1.65x10 ¹³ vg/mL
AAV8	hDes	sKL	6.5x10 ¹³ vg/mL
AAV8	hDes	sKL	2.5x10 ¹³ vg/mL
AAV8	hDes	sKL	1.03x10 ¹³ vg/mL
AAV8*	hDes	sKL	7.72x10 ¹² vg/mL
AAV8	hDes	sKL	2.13x10 ¹³ vg/mL
AAV8	hDes	sKL	9.31x10 ¹² vg/mL
AAV8	hDes	sKL	1.56x10 ¹³ vg/mL
AAV8	hDes	sKL	3.44x10 ¹³ vg/mL
AAV8	hDes	Null	4.33x10 ¹³ vg/mL

AAV8	hDes	Null	1.15x10 ¹³ vg/mL
AAV8	hDes	Null	2.18x10 ¹³ vg/mL
MyoAAV	hDes	sKL	1.07x10 ¹³ vg/mL
MyoAAV	hDes	sKL	1.17x10 ¹³ vg/mL
MyoAAV*	hDes	sKL	3.56x10 ¹³ vg/mL
MyoAAV	hDes	sKL	2.45x10 ¹³ vg/mL
MyoAAV	hDes	sKL	1.73x10 ¹³ vg/mL
MyoAAV	hDes	sKL	3.32x10 ¹³ vg/mL
MyoAAV	hDes	sKL	2.05x10 ¹³ vg/mL
MyoAAV	hDes	sKL	2.93x10 ¹³ vg/mL
MyoAAV	hDes	Null	1.56x10 ¹³ vg/mL
MyoAAV	hDes	Null	1.01x10 ¹³ vg/mL

*Aliquots generated by concentrating low titer productions with Amicon 100 kDa centrifugal filter units (Sigma).

2.1. Generation of AAV vectors

2.1.1. Triple transfection and cell harvest

AAV vectors were produced with the triple transfection method, as outlined by Piedra et al. (2015). The method involves the simultaneous transfection of pXX6, pRep2CapX, and the plasmid with the expression cassette into HEK293 cells. pXX6 contributes to adenoviral helper genes; pRep2Cap9 is essential for viral replication and capsid formation, with the Cap genes depending on the serotype (Cap8, Cap9, or CapMyo); and the plasmid with the expression cassette (or null sequence) is flanked the ITR sequences.

The transfection mixture was created by diluting pXX6, pRep2CapX, and the plasmid with the expression cassette in a ratio of 2:1:1, using 0.9% NaCl (B. Braun). Simultaneously, a PEI solution was prepared by adding 2.25 μ L of concentrated PEI (PolyScience) per μ g of DNA. The DNA solution was slowly vortexed with the diluted PEI and incubated for 20 minutes at room temperature to allow for PEI to complex with the DNA plasmid. Subsequently, the required amount of the transfection mixture was introduced into 15 cm culture plates containing HEK293 cells with a confluency of approximately 80-90%, along with 12 mL of transfection media.

After a 6-hour incubation at 37°C and 5% CO₂ in an incubator, the transfection media was replaced with growth media to prevent PEI-induced toxicity. After 48-78 hours cells were detached from the plates using a scraper (Falcon) and transferred to a 50 mL tube. Following a 15-minute centrifugation at 200g, the culture media (supernatant) and cell pellet were separated for

independent virus purification. The supernatant was stored at -20°C, while the cell pellet was resuspended in lysis buffer and stored at -80°C until virus purification.

Transfection media: DMEM (Corning), 1% FBS (LabClinics), 1% P/S (LabClinics)

PEI 10x: 100mM Polyethylenimine Mw 25.000 (Sigma) in dH₂O, adjust to pH=7 with HCl 37% (Panreac). Filtered with a 0,22 µm Stericup (Merck).

Lysis buffer: Tris 50 mM (Trizma, Sigma), NaCl 20 mM (Panreac), MgCl₂ · 6H₂O 2 mM (Panreac). HCl 37% to adjust pH at 8.5, dH₂O. Autoclaved.

2.1.2. AAV purification

AAV particles within cells were extracted by lysing cell pellets by freeze/thaw cycles. Following a 10-minute centrifugation at 2,000g, cellular debris was discarded, and the supernatant was treated with 50U/mL of benzonase (Sigma) for 1 hour at 37°C. This enzyme degrades non-encapsidated DNA, which could interfere with the final AAV quantification. The solution was then centrifuged for 20 min at 3,000g, and 1 mL of 40% PEG (Sigma) per mL of cellular lysate was added together with NaCl to a final concentration of 200mM. The resulting solution was incubated overnight to allow AAV virions to precipitate. The AAVs were precipitated by a 15-minute centrifugation at 8,000g and 4°C, and the resulting pellet was resuspended with 5 mL of lysis buffer.

Simultaneously, AAV particles present in the supernatant were precipitated by adding the same proportions of 40% PEG and NaCl, and incubated overnight at 4°C. The precipitated solutions were then centrifuged for 30 min at 3,000g and 4°C, and the resulting pellet was resuspended with 5 mL of lysis buffer. Subsequently, 50U/mL of benzonase (Sigma) was added, and the mixture was incubated for 1 hour at 37°C. Finally, the resulting solutions from the cell pellet and the supernatant precipitates were combined and further purified by an iodixanol gradient.

The iodixanol gradient was created with solutions with different percentages of iodixanol: 15% diluted with PBSMK-NaCl, 25% diluted with PBSMK and 20 µL of phenol red, 40% diluted with PBSMK, and 60% diluted with PBSMK and 2 µL of phenol red. Phenol red allows color differentiation of the different gradient phases, serving as a visual confirmation of proper gradient formation. These solutions were sequentially added into an ultracentrifugation polypropylene tube (Beckman Coulter), starting with less dense solutions, and finally adding the AAV solution on top. The gradient was then centrifuged at 69,000 rpm in a Beckman 70Ti rotor (Beckman Coulter) for 1 hour and 30 minutes at 18°C. The AAV density band in the gradient was collected into different aliquots.

Phosphate buffered saline (PBS): 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na₂HPO₄ (Alco), and 1.8 mM KH₂PO₄ (Alco), pH = 7.4.

PBSMK: 137 mM NaCl, 5.5 mM KCl, 10 mM Na₂HPO₄ · 12H₂O, 1.7 mM KH₂PO₄ (Alco), 1 mM MgCl₂ · 6H₂O (Panreac) in dH₂O. Adjusted to pH=7.4 with HCl 37% (Panreac). Filtered with a 0.22 µm Stericup.

PBSMK 1M NaCl: 1M NaCl, 5,5 mM KCl, 10 M Na₂HPO₄ · 12H₂O, 1.7 mM KH₂PO₄, 1 mM MgCl₂ · 6H₂O in dH₂O. Adjusted to pH=7.4 with HCl 37%. Filtered with a 0.22 µm Stericup.

2.1.3. AAV quantification

The AAV aliquots were quantified using the Quant-iT PicoGreen (ThermoFisher) method as previously described (Piedra et al., 2015). This molecule intercalates with double-stranded DNA (dsDNA) and generates a fluorescent signal directly proportional to the amount of dsDNA present in the solution. For quantification, 2 µL of the AAV solution was combined with either 18 µL of PicoGreen lysis buffer or 18 µL of TE buffer. The mixture with lysis buffer was incubated in a thermocycler (Eppendorf) for 1 hour at 70°C, followed by gradual cooling at a rate of 5°C per minute until 25°C. The resulting solutions were transferred to a black 96-well plate, and 180 µL of PicoGreen dilution (1:600) was added. Finally, the fluorescence was measured using a Spark plate reader fluorimeter (Tecan). To infer the encapsulated AAV genomes, the obtained fluorescence values were compared to a standard curve of known dsDNA concentrations. The fluorescence value of each non-lysed sample was subtracted from that of the lysed sample to eliminate background and impurities noise. The final viral genome titer data were obtained using the following formula:

$$vg/mL = \frac{A(ng/mL) \cdot 1,82 \times 10^{12}(bp/ng)}{B(bp/vg)}$$

where A = ng is the DNA concentration and B = length of the AAV genome (bp between ITR). The correction factor of 1.82×10^{12} considers that strands from different AAV particles are hybridized.

PicoGreen lysis buffer: Tris-HCl 11mM (Sigma, Panreac), SDS 0.11% (USB), NaCl 110 mM (Panreac) in ddH₂O.

TE buffer: 10mM Tris-HCl (Sigma, Panreac), 1mM EDTA (USB) in ddH₂O.

2.1.4. Sterility and mycoplasma tests

To detect various types of bacterial contaminations, 2 µL of the final AAV aliquots were inoculated in the following media and examined for contamination the following week:

- Growth medium without antibiotic, placed in the incubator at 37°C.
- Fluid Thioglycollate Medium (FTM, Merck) in a 15 mL tube. Incubated at 32.5°C.
- Tryptic Soy Broth (TSB, Sigma) in a 15 mL tube. Incubated at room temperature.

Fungi contamination can be examined in the first condition. For assessing *Mycoplasma spp.* contamination, 2 µL of the final AAV aliquots were inoculated in the growth medium of a 10cm plate (Falcon) during A549 cell passage. An extra cell passage was performed and one week later 200 µL were recovered. The sample was boiled at 95°C for 10 min and centrifuged at maximum speed for 5 min to pellet cellular debris. Two microliters are used directly for PCR using the Venor®GeM Classic kit (Minerva biolabs). The following PCR program was performed: hot start of 95°C for 2 min; 40 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s. PCR products were checked in a 2% agarose gel loaded with Xylene Cyanol. Positive control and samples with *Mycoplasma spp.* show a band of 265-278 bp, while negative samples and internal control show a band of 191 bp.

TAE 1x: 40mM Tris-Ac pH 8.0

Loading buffer 10x: 50% Glycerol (Panreac), 100 mM EDTA pH 8.0 (USB), 1% SDS (USB), 0.1% Bromophenol blue (Sigma), 0.1% Xilencianol (Sigma).

3. *In vitro* cultures

3.1. Cell lines

3.1.1. Cell seeding

The HEK293 AAV cell line (Agilent) was used for AAV vector production. The C2C12 myoblast cell line was used for confirming expression from Desmin constructs. The A549 cell line was used to detect mycoplasma contamination of viral preparations. Frozen cell vials were thawed in a 37°C water bath. cells were grown in culture media on 15 cm plastic plates (Falcon) within cell incubators (Forma Scientific) maintained at a constant temperature of 37°C with 5% CO₂ and 95% humidity.

Growth media: Dulbecco's Modified Eagle Medium with 4.5 g/L of glucose (Coring), 10% v/v Fetal Bovine serum (FBS, Labclinics), 1% v/v Penicillin-streptavidin antibiotic solution (PS, Labclinics)

3.1.2. Cell passage and maintenance

Upon reaching a high cell confluency, exceeding 75% of the plate surface covered by cells, mechanical detachment of cells from the plate was performed by resuspending them in media. The cell suspension was then transferred to a 50 mL tube and centrifuged for 5 min at 200g to pellet the cells. The supernatant was discarded, and the cells were resuspended in fresh growth media. The desired volume of the cell suspension was then transferred to new plates containing up to 17 mL of fresh growth media for cell line amplification and maintenance. The C2C12 cell line was grown in the same conditions, but cells were split at 50-70% to avoid fusion and differentiation by contact. Cells were incubated again at the incubator.

3.1.3. Cell freezing

Cells were detached from plates, transferred to a 50 mL tube, and centrifuged for 5 min at 200g. Cell pellets were resuspended in 1 mL of freezing media and transferred to a cryotube. Cryotubes were set in a controlled rate freezing chamber inside the -80°C freezer.

Freezing media: FBS (Labclinics), 10% v/v dimethyl sulfoxide (DMSO, Sigma)

3.2. Spinal cord organotypic cultures (SCOCs)

SCOCs were prepared following the previously described method (Rothstein et al., 1993). P8 Sprague-Dawley rats were used and following euthanasia, the spinal cord was aseptically harvested and immersed in ice-cold Gey's Balanced Salt Solution (GBSS) with high glucose content (6.4 mg/mL), and meninges were carefully removed. The spinal cord was then transversely cut into 350 µm thick slices using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co., Surrey, UK). L4-L5 lumbar sections were delicately transferred onto Millicell-CM porous membranes (0.4 mm; Millipore, Burlington, MA, USA) in a six-well plate containing 1 mL of incubation medium.

During the initial week, the cultures were allowed to stabilize, as a substantial number of neurons naturally die, and glial cells exhibit a strong reactivity due to the axotomy performed during the culture procedure. At 7 days in vitro (DIV) viral vectors were either added to the media or in a one-microliter drop on top of the sections. Infection with Ad vectors was performed either with 10^6 or 10^8 total IU in the drop or with 10^6 or 10^8 IU/mL in media. For AAV vectors, the titers were of 10^{10} vg contained in the one-microliter drop. Simultaneously, some slices were treated with a drop of PBS or were left untouched. At 14 DIV the cultures were either fixed with 4% paraformaldehyde (PFA, Casa Álvarez) for an hour and processed for transduction studies, or exposed to glutamate. Acute glutamate exposure was performed for 30 min by replacing the growth media with 50mM glutamate in Locke's solution. At 15 DIV sections were fixed in 4% PFA for MN survival analyses. To compare viral vector transductions, experiments were performed in parallel on sections plated the same day with 5-10 replicates for each condition. To assess MN survival SCOCs were derived from at least three independent cultures conducted on different days, resulting in 17-25 slices for each condition.

Incubation media: 50% minimal essential medium (MEM), 25% heat-inactivated horse serum, 25 mM HEPES, 2 mM glutamine, and 25% Hank's Balanced Salt Solution (HBSS) supplemented with 25.6 mg/ml glucose; pH 7.2.

Locke's solution: 137mM NaCl, 2.5 mM CaCl₂, 5 mM KCl, 5.6 mM D-glucose, 0.3 mM KH₂PO₄, 4 mM NaHCO₃, 0.3 mM Na₂HPO₄, 0.01mM glycine and 10 mM HEPES prepared in distilled H₂O and filtered.

4. Animal experiments

4.1. Animal housing

Transgenic mice expressing the G93A mutation in the human SOD1 gene and non-transgenic wild-type (WT) littermates with the B6SJL/J background were used for the characterization and gene therapy studies. High-copy number SOD1^{G93A} mice (Tg[SOD1-G93A]1Gur) were obtained from The Jackson Laboratory in Bar Harbor, ME. These mice were bred by mating transgenic males with F1 hybrid females from Janvier Laboratories in France and maintained as hemizygotes. P8 wild-type Sprague-Dawley rats were used in the spinal cord organotypic cultures studies. Animals were housed under standard conditions with unrestricted access to food (Teklad 2018) and water at the animal services of the Universitat Autònoma de Barcelona. Proper care and handling of the animals was carried out following European Union Council Directives (Directive 2010/63/EU) and Spanish regulations for the use of laboratory animals. The experimental procedures were approved by the Ethics and the Biosafety Committees of the Universitat Autònoma de Barcelona (CEEAH 4812 and HR-128-18).

4.2. hSOD1^{G93A} mice genotyping

4.2.1. Conventional PCR genotyping

Transgenic mice were identified by polymerase chain reaction (PCR) amplification of DNA extracted from the tail. Tails were digested overnight with NaOH 50 mM at 55°C, vortexed to disaggregation, and centrifuged at maximum speed for 10 min. Two microliters of the supernatant were mixed in the PCR reaction containing DreamTaq 1x buffer (ThermoFisher, B65), 10 pmol/μl of each primer, 25 mM dNTPs (ThermoFisher, R0191), and 1.25 units of Dream Taq polymerase (ThermoFisher, EP0702) to a final volume of 25μl. The following PCR program was used (Eppendorf Mastercycler gradient): heating 95°C for 3 min, denaturation of DNA strands at 95°C for 30s, primer annealing at 62°C for 30 s and elongation at 72°C for 45s. Repetition of the second to fourth step 34 times.

The products were mixed with xylene cyanol loading buffer and checked in a 1,5% agarose (Amresco)/TAE gel with the GeneRuler™ 1 kb DNA ladder (ThermoFisher). The gel was run in an electrophoresis cuvette for 60 min at 120V and visualized under a UV transilluminator (Syngene).

Table 8. Primers for PCR hSOD1^{G93A} mice genotyping

PCR primers	Product size (bp)	Forward	Reverse
Human SOD1	236	CATCAGCCCTAATCCATCTGA	CGCGACTAACAATCAAAGTGA
Mouse IL-2	324	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC

TAE buffer 50X: 40 mM Tris (Sigma), 20 mM glacial acetic acid (Panreac) and 0.5M EDTA pH 8 (USB) in ddH₂O, autoclaved.

4.2.2. Digital PCR for hSOD1^{G93A} copy number quantification

For copy number determination genomic DNA samples were diluted to 15 ng/μl and digested with HindIII or BstYI restriction endonucleases, added at 10 U/μg gDNA with 1X NEBuffer (New England Biolabs) and incubated following manufacturer's instructions for an hour. The reaction mixes were prepared for the target gene (hSOD1) and the reference gene (mIL2) separately. This contained 1x QX200 ddPCR EvaGreen (Bio-Rad), 200nM of each primer and 3.3. ng of gDNA. Each sample, consisting of 22 μl reaction mix and 22 μl gDNA, was loaded in 20 μL duplicates in a DG8™ Cartridge (Bio-Rad), followed by the addition of 70 μl of QX200 Droplet Generation Oil for EvaGreen (Bio-Rad).

Droplets were generated in a QX200 Droplet Generator (Bio-Rad), transferred to a 96-well plate, and heat sealed with pierceable foil on a PX1 PCR Plate Sealer (Bio-Rad). Thermal cycling followed (T100 thermocycler, Bio-Rad) with a reaction volume of 40 µl and the following setup: heating 95°C for 5 min, denaturation at 95°C for 30s, primer annealing/extension at 58°C for 1min and signal stabilization at 4°C for 5min followed by 90°C for 5min. Forty cycles were performed with a 2°C/sec ramp between steps. Data acquisition was performed using the QX200 Droplet Reader and QuantaSoft™ Software (Bio-Rad), with concentrations reported as copies/µl. The copy number variation (CNV) was calculated as the ratio of the concentration of hSOD1/mL2 and normalized for the number of copies of the reference gene in the genome.

Table 9. Primers for ddPCR hSOD1^{G93A} copy number determination

ddPCR primers	Forward	Reverse
Human SOD1	GCACACTGGTGGTAAGTTTTCAT	CGCGACTAACAAATCAAAGTGA
Mouse IL-2	GGGTCTGTGCATTAGTCAGC	GTAGGTGGAAATTCTAGCATCATCC

4.2.3. Real-time PCR for hSOD1^{G93A} copy number verification

For routine CNV verifications, RT-PCR was performed following a protocol previously described (Alexander et al., 2004). The real-time amplification was carried out in a 10 µl/well volume, comprising 1x SYBR green (Bio-Rad), 0.4 µM of each primer aliquot, and dH₂O. Two ng of DNA were added in 2 µl. Known copy samples serve as standards on the same plate for accurate quantification. Assays were conducted in duplicate on a real-time CFX96 qPCR thermocycler (Bio-Ras), involving 40 cycles of amplification at 95°C for 10s and 58°C for 30s after the initial Taq polymerase activation at 95°C for 3 min. Subsequently, primer melt analysis was performed. The CT values were subtracted, and ΔCT for each animal was computed:

$$\Delta CT = CT_{mIL2} - CT_{hSOD1}$$

To correct for differences in amplification, the ΔCT was normalized using a formula that incorporates average CT values and the slope of the CT_{mIL2} vs. ΔCT plot. The absolute copy number of the mutant hSOD1 was then evaluated using the formula: $\Delta CT = \Delta CT + (AV_{CT_{mIL2}} - CT_{mIL2}) \times \text{Slope}^{CT_{mIL2} \text{ vs. } \Delta CT}$

where $AV_{CT_{mIL2}}$ = mice average mL2 CT for a given amount of DNA load and;

$\text{Slope}^{CT_{mIL2} \text{ vs. } \Delta CT}$ = slope of the CT_{mIL2} vs. ΔCT plot for a given transgene copy number

To apply these corrections a record of values for mice with the same transgene copies must be kept. Transgene copies can be estimated as long as the absolute copy number of any mouse is known (ddPCR):

$$\text{Transgene copies} = \text{KCN} \times 2^{\Delta\Delta\text{CT}}$$

where KCN = known absolute copy number of a given animal

$$\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{unknown}} - \Delta\text{CT}_{\text{known}}$$

Table 10. Primers for RT-PCR hSOD1^{G93A} copy number verification

qPCR primers	Forward	Reverse
Human SOD1	CATCAGCCCTAATCCATCTGA	CGCGACTAACAATCAAAGTGA
Mouse IL-2	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC

4.3. Surgical procedures

4.3.1. Brachial plexus injury model

A brachial plexus transection was performed affecting the three trunks of the nerve. B6SJLF1/J mice were anesthetized with a ketamine (Ketamidol, Ritcher Pharma) and xylazine (Rompun, Bayer) mix (100/10 mg/kg), placed in a supine position and shaved. A longitudinal incision near the clavicle exposed the left brachial plexus. Careful dissection involved cutting the three nerve trunks close to the ganglion without harming subclavian vessels. Nerve ends were separated to prevent regeneration. The muscles and the skin were sutured (6/0 nylon sutures, B. Braun) and the animals recovered for 1-2h on a heating pad. Buprenorphine (0.3 mg/mL Bupaq, Ritcher Pharma) was administered subcutaneously (0.1 mg/kg) during 48h. The animals were supervised daily until euthanasia. The contralateral forelimb muscles and hemi-spinal cord were used as controls for the study.

4.3.2. Viral vector intrathecal and intravenous administrations

Animals were administered with intrathecal or intravenous injections. Intrathecal lumbar administrations were performed as previously described (Homs et al., 2014). Briefly, mice were induced into an anesthetic plane for surgery with 4% isoflurane (ISOFLOR 100% p/p, Zoetis) and maintained with 2% of the inspired air.

Mice were immobilized in a prone position and shaved. Following spine exposure through paravertebral muscle dissection, a 33-gauge needle, and a Hamilton syringe were used to slowly inject viral vectors in the CSF between lumbar vertebrae L3 and L4. A total of 2×10^{11} vg of AAV9-CAG-sKL were infused in 10 μ L. Verification of proper access to the intrathecal space was confirmed by observing the animal's tail flick. The needle was left in position at the injection site for an additional minute to avoid reflux before suturing the muscle with absorbable sutures (5/0 Safil® sutures, B. Braun) and the skin with Michel suture clips (12040-01, F.S.T.). Suture clips were removed one week after surgery.

For intravenous injections viral vectors were diluted with NaCl 0.9% (B. Braun) to a final volume of 200 μ L. Animals were previously warmed in a heating pad, immobilized in a restrainer and the tail was heated with an infrared light to induce vasodilation. Viral preparations were injected manually with a 30G needle attached to the syringe (ICOPLUS3, Sterican 30G 1/2" B. Braun) into the lateral tail vein of the mice.

4.4. Electrophysiological tests

4.4.1. Nerve conduction tests

Electrophysiological follow-up of the animals was carried out at 8, 12, and 16 weeks of age for those treated during the presymptomatic stage. For animals treated at the symptomatic stage, the monitoring occurred every two weeks starting from the 8th week. Animals were anesthetized with sodium pentobarbital (Sigma) at 40-50 mg/kg, immobilized in a prone position, and kept on a thermostated plate. For motor nerve conduction tests, the sciatic nerve was stimulated percutaneously using single pulses of 20 μ s duration (Grass S88) delivered by two needle electrodes positioned at the sciatic notch (Fig. 56), with increasing intensity until a maximal response was recorded. The transmission of impulses of α -motor axons initiates synaptic transmission at the NMJ, leading to the generation of an action potential in the membrane of skeletal muscle fibers. This was captured as the compound muscle action potential (CMAP, M wave) by microelectrodes carefully positioned under a magnifying glass at the tibialis anterior (TA) and plantar interosseus (PL) muscles (Mancuso et al., 2011; Navarro & Udina, 2009).

To assess the central descending motor pathways, motor evoked potentials (MEP) were recorded at the TA muscles. This was achieved by applying transcranial electrical stimulation to the motor cortex using 0.1 ms individual rectangular pulses at maximal intensity. Stimulation electrodes were inserted subcutaneously above the skull, with the cathode covering the sensorimotor cortex and the anode situated at the nose.

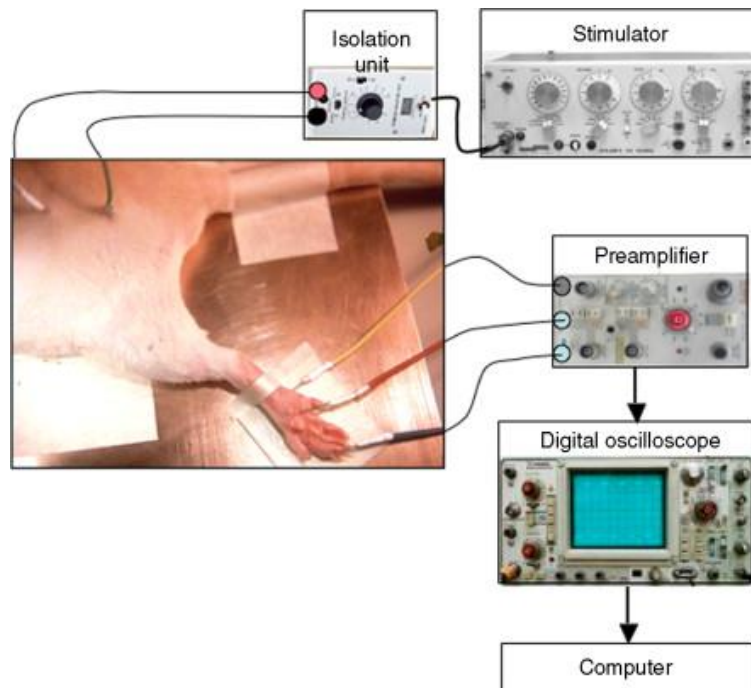


Figure 56. Elements of an electrophysiological system for assessing nerve conduction. The image shows the positioning of stimulation electrodes at the sciatic notch, along with recording electrodes at the PL muscles and ground electrodes placed at the hind paw of a rat. (Extrated from Navarro & Udina, 2009)

The MEP/CMAP amplitude ratio was calculated to assess the relative contribution of UMN involvement in the overall motor pathway. As the recording of MEP in the muscle depends on LMN synapsis, the ratio may help distinguish between the relative involvement of UMNs and LMNs and correct the MEP values according to the CMAP values. All potentials were amplified (x100 to x1000), band-pass filtered between 10Hz and 2KHz and displayed on a digital oscilloscope (Tektronix 450S). Parameters assessed were the amplitude from baseline to the maximum negative peak and the latency from stimulus to the onset of the initial negative deflection and to the end of the wave (García-Alías et al., 2003; Mancuso et al., 2011).

4.4.2. Motor unit number estimation (MUNE)

For the motor unit number estimation (MUNE) the incremental technique protocol was followed (Mancuso et al., 2011; Shefner et al., 2006), employing the same parameters as in the motor nerve conduction tests. The MUNE process involved stimulating the sciatic nerve starting with subthreshold intensity and applying pulses of gradually increasing intensity, recording quantal increases in CMAP. To prevent the alternation phenomenon, where the combination of already recruited MU alters the CMAP instead of the recruitment of new MU, only increments exceeding 50 μ V were considered. The mean amplitude of individual motor units (SMUA) was computed as the average of consistent increases. The estimated number of MU was then determined using the equation: $MUNE = CMAP \text{ maximal amplitude} / SMUA$.

4.5. Behavioral tests

4.5.1. Rotarod test and clinical disease onset

The Rotarod test was conducted to assess locomotor coordination, strength, and balance of the animals, as previously outlined (Mancuso et al., 2011). Mice were placed on a rotating rod (Ugo Basile®) at a constant speed of 14 rpm, and the duration each animal remained on the rod was measured. Five trials were given to each mouse, and the longest time until falling was recorded, with a cut-off time of 180 seconds.

Mice were trained for three consecutive days the week before the experiment. The training began at 8 rpm and progressively increased to 11 rpm on the second day, followed by an increase to 14 rpm on the last day. This test was performed weekly from 8 to 16 weeks of age. The clinical onset of disease for each animal was identified as the first week in which the cut-off time consistently fell below 180 seconds.

4.5.2. Grip strength test

To evaluate limb grip strength, mice were positioned on a metallic grid, permitting all four paws to grip the bars while gently pulled parallel by the tail. The Grip Strength Meter (Ugo Basile®) recorded the peak pull force just before the mouse released from the grid during the test. Each animal underwent a minimum of three trials with rest intervals in between, and the apparatus was cleaned with ethanol after each trial. The recorded measurement represented the maximum force exerted, expressed in grams.

4.5.3. Treadmill test

For the treadmill running test, mice at 16 weeks of age were placed on a motor-driven 5-lane treadmill (Panlab, Harvard apparatus) for 20 minutes to acclimate them to the device and the shock stimulus. During acclimatization sessions, the shock grid intensity was set at 0.1 mA to provide a mild negative stimulus. The protocol consisted of a single treadmill running session, starting at 5 cm/s and gradually increasing to 30 cm/s over 5 minutes. The maximum speed at which the animals ran before consistently encountering the shock grid was recorded.

4.5.4. Body weight and clasping monitoring

Body weight changes in the animals were monitored weekly. This procedure was conducted prior to motor and electrophysiological tests to prevent subtle variations in weight caused by stress or anesthesia. Simultaneously, the mice clasping behavior was observed by gently lifting the mouse by the tail and holding it in a vertical position. The clasping response involves the mouse bringing its hindlimbs toward the abdomen. The clasping score was evaluated as follows: score 0 indicated no clasping, score 1 represented slight clasping or clasping of one leg, score 2 denoted moderate clasping during tail suspension, and score 3 indicated severe clasping with hindlimb retraction, even when the mouse was placed on a flat surface. However, only the onset time of the clasping was reported in the results (Hatzipetros et al., 2015).

4.5.5. Survival analysis

To evaluate survival, groups of animals were observed until they reached a humane endpoint. This endpoint was defined by loss of the righting reflex, indicating the moment when a mouse could not turn and sustain an upright standing position for 30 seconds when placed on its side (Ludolph et al., 2010).

4.6. Animal euthanasia, perfusion, and sample processing

Animals were euthanized by an overdose of sodium pentobarbital (Sigma, >200 mg/kg) and decapitated for fresh sample extraction. Mice from the characterization and gene therapy studies were sacrificed at 16 weeks, while animals with BPI were sacrificed at 7- and 28-days post-injury (dpi). Samples were quickly extracted under a dissecting binocular magnifying glass and freshly frozen in liquid nitrogen. For histological analysis, the animals were transcardially perfused with PFA 4% in PBS. Mice were deeply anesthetized with either a mixture of ketamine (Ketamidol, Ritcher Pharma) and xylazine (Rompun, Bayer) (100/10 mg/kg) or with sodium pentobarbital (100 mg/kg). Perfusion was conducted once the animals no longer displayed pedal reflex. A cut was made in a liver lobe, and a solution of cold 4% PFA was gradually intracardially infused into the right ventricle. This process serves to flush the blood through the liver and fixate the tissues. Following a 10-minute perfusion, the samples were collected. The spinal cords underwent post-fixation in cold 4% PFA in PBS for 2 hours, followed by PBS washes and subsequent embedding in a 30% sucrose solution. Muscle samples were directly embedded in the 30% sucrose solution to prevent any additional autofluorescence of the tissue.

Sucrose 30%: 30% w/v of sucrose (Fisher Scientific) in PBS 1x with 0.05% sodium azide (Sigma).

5. Histological analysis

Perfused tissue samples in the 30% sucrose solution were kept at 4°C for a minimum of two weeks until they sank to ensure cryoprotection. Tissues were embedded in OCT (Tissue-Tek) and rapidly frozen on dry ice or in the cryostat chamber (Leica). For the spinal cord, the lumbar region was transversally cut at a thickness of 20 µm, collected in a sequential series of 10 slides, and stored at -20°C. For the muscle, transversal sections separated by 100 µm were cut at 10 µm. For free-floating staining, muscle samples were longitudinally sectioned at a thickness of 50 µm for NMJ analysis. Coronal brain sections were cut at 40 µm and serially collected for the analysis of cortical MNs. Free-floating sections were either collected in a PBS-filled plate for subsequent immunohistochemistry or preserved in an anti-freeze solution and stored at -20°C.

Anti-freeze solution: 20% w/v sucrose (Fisher Scientific), 1% w/v Polyvinylpyrrolidone (Sigma), 30% w/v Ethylenglycol (Sigma) in dH₂O

5.1. Cresyl violet of spinal cord sections

Slides corresponding to L4-L5 lumbar spinal cord sections separated 100 µm were stained in cresyl violet solution for 3h. The samples were dehydrated using solutions with increasing ethanol (Scharlab) concentration after which they were placed in xylol (Panreac) for 2 min. Slides were mounted with coverslips and DPX mounting medium (Sigma).

Spinal cord sections were analyzed under a microscope (Nikon Eclipse Ni) and representative photos were taken. MNs were identified by their localization in the ventral horn and following strict size and morphological criteria. Only neurons with a diameter larger than 20 µm, polygonal shape, and prominent nucleoli were counted.

Cresyl violet solution: 3mM cresyl violet acetate (Sigma) are dissolved in dH₂O and 5 drops of glacial acetic acid (Panreac) are added for each 200 mL of solution.

5.2. Immunohistochemistry

5.2.1. SCOCs immunofluorescence staining and analysis

SCOC slices from various experimental conditions were fixed using 4% paraformaldehyde in PBS for 1h at room temperature. Following this, slices were blocked with 5% normal horse serum (Sigma) and in TBS-TX. Subsequently, sections were incubated for 48 hours with primary antibodies, specifically anti-neurofilament H non-phosphorylated (SMI-32, 1:1000, BioLegend), anti-Klotho (1:100, BAF1819, RDSsystems) and anti-Iba1 (1:1000, 19-1974, Rafer, Fujifilm) or anti-GFAP (1:1500, Z0334, DakoCytomation). After thorough washing in TBS-T, the slices underwent incubation with the corresponding secondary antibodies diluted in blocking buffer. The secondary antibodies for the transduction studies with GFP were Alexa Fluor 647 donkey anti-mouse IgG (1:500; ab150075, Abcam) and Cy3-conjugated antibody donkey anti-mouse IgG (1:500; 715-165-151, IR). For assessing MN survival, the secondary antibodies used were Alexa Fluor 647 donkey anti-mouse IgG (1:500; ab150075, Abcam), Alexa Fluor 488 donkey anti-mouse IgG (1:500; A21202, Invitrogen) and Alexa Fluor 594 donkey anti-goat IgG (1:500; A11058, Invitrogen). Finally, cell nuclei were labeled with DAPI (1:2000) for 1 min in TBS, and the sections were mounted with Fluoromount-G medium (SouthernBiotech). The analysis of the slides was performed using confocal microscopy and Fiji software. For GFP co-labeling evaluation the colocalization ImageJ plugin was used and reported as % of the volume. SMI-32 positive cells in the ventral horn of each hemislice were quantified using the Cell Counter tool in ImageJ software (NIH).

5.2.2. Immunohistochemistry of spinal cord slices

Spinal cord sections were permeabilized with TBS-TX for 10 min and underwent blocking with 10% normal donkey serum (NDS) solution for an hour. Slices were incubated in blocking solution for 24 hours at 4°C with primary antibodies. The primary antibodies used were anti-Iba-1 (1:500; 19-19741, Rafer, Fujifilm) or anti-GFAP (1:500, 130300; Invitrogen) with anti-Vimentin (1:600, ab92547, Abcam). Following TBS-T washes, sections were then incubated overnight with the corresponding secondary antibodies: Alexa Fluor 488-conjugated secondary antibody (1:600; A21206, Invitrogen) or Cy3-conjugated secondary antibody (1:200; 712-165-150, Jackson IR). Finally, slices were washed again with TBS-T and mounted in DAPI Fluoromount-G (ThermoFisher) for staining the nucleus. Labeling was examined under confocal microscopy (Zeiss LSM 700). For the assessment of astroglia and microglia immunoreactivity and vimentin staining, photographs of the ventral horn were captured at ×20 magnification. After setting a threshold for background, the integrated density of GFAP, Iba1, or vimentin labeling was measured using ImageJ software (NIH).

Tris-Buffered Saline (TBS): 0.05 M Tris (Trizma base, Sigma) and 0.15 M sodium chloride (Panreac), pH 7.6.

TBS-TX permeabilizing solution: 0.3% Triton X-100 (ThermoFisher) in TBS.

TBS-T washing solution: 0.1% Tween20 (ThermoFisher) in TBS.

Blocking solution: 10% normal donkey serum (Merck) in TBS, 0.3% Triton X-100 (ThermoFisher) and 0.2-mM glycine (Sigma)

5.2.3. Immunohistochemistry of muscles and KL signal quantification

To detect KL in the muscle samples, immunofluorescence was conducted on 10 µm transverse TA muscle sections. An in-house made rabbit antibody anti-KL (K113) (Massó et al., 2015), specific to the secretion signal of secreted KL, was used and the signal was amplified following the instructions of the Alexa Fluor™ 488 Tyramide SuperBoost™ Kit (B40932, Fisher Scientific). In brief, muscle slices underwent antigen retrieval in boiling citrate buffer for 30 minutes at room temperature. After three washes with PBS 1x, hydrogen peroxide solution was applied to inhibit endogenous peroxidase for 1 hour.

Following three additional washes with PBS 1x, the endogenous biotin-blocking kit was employed. Subsequently, the slices were washed with PBS-T, and blocking buffer was applied for an hour. Primary antibodies, anti-KL (in-house) and anti-collagen type IV (1:200, 134001, Setareh Biotech), were added with blocking buffer and left to incubate overnight. The next day, slides were washed with PBS-T and incubated with the secondary biotinylated horse anti-rabbit IgG (1:200, BA-1100, Vector) and Alexa Fluor 488 Donkey anti-Goat IgG (1:200, A11055, Invitrogen) for 90 minutes.

After washes with PBS-T, the slices were incubated with Streptavidin-HRP (Str-HRP) for 1 hour at room temperature, followed by additional washes to eliminate any remnants. For TSA (Tyramide Signal Amplification) development, 100 µl of tyramide working solution, prepared according to the manufacturer's instructions, was added to the slices. The reaction was stopped with the reaction stop reagent within 7 minutes.

After washing with PBS 1x, slides were mounted with DAPI Fluoromount-G (ThermoFisher). Images of the muscles were captured at x40 with confocal microscopy (Zeiss LSM 700) and the KL signal was quantified using ImageJ software (NIH) as the integrated density after setting a threshold for background.

Phosphate buffered saline (PBS 1x): 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na₂HPO₄ (Alco), and 1.8 mM KH₂PO₄ (Alco), pH = 7.4.

PBS-T washing solution: 0.1% Tween20 (ThermoFisher) in PBS 1x.

Citrate buffer: 82.5 mM sodium citrate dihydrate and 17.5 mM of citric acid, pH=6 adjusted with HCl (Panreac)

5.2.4. Immunohistochemistry and analysis of NMJ

Longitudinal muscle sections were washed with PBS1x for 10 min, incubated in boiling PBS1x for 5 minutes, and permeabilized for 10 min. Subsequently, the sections were blocked with NDS 5% for 1 hour at room temperature. The incubation with primary antibodies in blocking solution was carried over 48 hours at 4°C, with anti-NF200 (1:1000, AB5539, Merk), and anti-synaptophysin (1:500, ab32127, Abcam).

Following primary antibody incubation, the sections were washed 3 times for 10 minutes and incubated with secondary antibodies in blocking solution overnight at 4°C. The secondary antibodies employed were Alexa Fluor 594-conjugated Goat x Chicken (1:200, A11042, Invitrogen), Alexa Fluor 594-conjugated Donkey x Chicken (1:200, A21207, Invitrogen) and α -bungarotoxin Alexa Fluor 488-conjugated (1:650, B13422, Invitrogen). Antibody solution was filtered with a 0.22 μ m filter. Sections were washed with PBS-Tw 0.1%, followed by a wash with PBS for 10 min and another wash with PB for 10 min. Muscle sections were placed on slides with the aid of a brush, dried, and mounted with DAPI Fluoromount G medium (ThermoFisher). Images were obtained using a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss). The maximum projection images presented in this study were created from 1.5 μ m Z projections. To analyze NMJ, the determination of fully occupied endplates involved classifying each endplate as either occupied (when presynaptic terminals overlaid the endplate), or vacant (when presynaptic terminals were not within the endplate). A minimum of 4 fields were examined per muscle.

PBS 1x: 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na₂HPO₄ (Alco), 1.8 mM KH₂PO₄ (Alco), pH = 7.4.

PBS-Tx permeabilizing solution: 0.3% Triton X-100 (ThermoFisher) in PBS 1x.

PBS-Tw washing solution: 0.1% Tween20 (ThermoFisher) in PBS 1x.

Blocking solution: 5% Donkey serum and 0.3% Triton X-100 in PBS 1x

5.2.5. Immunohistochemistry of brain sections and cortical neuron quantification

Brains were coronally cryosectioned at a thickness of 40 μm , starting from the olfactory bulb. The region of interest, corresponding to the primary motor cortex, was defined using a Mouse Brain Atlas in Stereotaxic Coordinates (Paxinos & Franklin, 2004), employing the union of the corpus callosum and the fusion of the ventricles as landmarks (from Bregma 1.10 to -0.22 mm). Within this region, five sections spaced 200 μm apart were subjected to immunostaining. Sections underwent permeabilization with PBS-Tx for 5 minutes and were subsequently blocked for 1 hour at room temperature. Primary mouse antibody against NeuN (diluted at 1:1000, MAB377, Merck) was prepared in 5% NGS in PBS-Tx and incubated at 4°C for 48 hours with constant agitation. Following incubation, sections were washed thrice for 5 minutes with PBS-Tx and incubated with secondary antibody goat anti-mouse Alexa 488 (1:1000, A11001, ThermoFisher) diluted in PBS-T 5% NGS at room temperature for 2h. For nuclear contrast, Hoechst (diluted at 1:10000, 33342, ThermoFisher) in PBS 1x was applied for 5 minutes. After four additional washes, slices were transferred to slides, air-dried for an hour, and mounted using Fluoromount G medium (ThermoFisher).

Confocal microscopy (LSM 980 Airyscan 2, Carl Zeiss) was used to capture tile images of the M1 cortex region. Imaris Software (version 9.2) was employed to analyze the image stacks from each hemisphere. The region corresponding to layer V was manually chosen, and a 3D surface of neurons was generated for quantifying neuron volume, surface area, and numbers. Quantification was performed on four sections per mouse, and neurons or stains with an area smaller than 100 μm^2 were excluded. Statistical analysis, following the approach outlined by Becker et al. (2017), involved the use of linear mixed models (LMM) implemented with the lme4 R package. This method was applied to assess whether genotype and treatment influenced cortical layer V neuron density. LMM addresses the challenges associated with statistically comparing groups with limited sample sizes and appropriately considers multiple measurements originated from the same mouse. A likelihood ratio test was conducted to compare the null hypothesis, comprising random effects terms for mouse and anatomical section, with the alternative hypothesis, which integrated these terms along with fixed effects for genotype and treatment.

PBS 1x: 137 mM NaCl (Panreac), 2.7 mM KCl (Panreac), 10 mM Na_2HPO_4 (Alco), 1.8 mM KH_2PO_4 (Alco), pH = 7.4.

PBS-Tx permeabilizing and washing solution: 0.2% Triton X-100 (ThermoFisher) in PBS 1x.

Blocking solution: 10% Normal goat serum (NGS, Sigma) and 0.2% Triton X-100 in PBS 1x.

6. ELISA

6.1. Protein extraction for ELISA

Gastrocnemius muscle and spinal cord samples were weighted and 5x volumes of lysis buffer were added. Tissues were disrupted in ice using a small dispersing instrument (IKA™ Disperser ULTRA-TURRAX™ T10 Basic), avoiding overheating until samples were completely homogenized. Samples were maintained in ice for 2h and centrifuged for 5 min at 14,000g at 4°C. The supernatant was collected into a new tube for further analysis.

Lysis buffer: 25mM HEPES (Sigma), 0.1% Igepal (Sigma), 5mM MgCl₂ (Merck) 1.3mM EDTA (Sigma), 1mM EGTA (Sigma), Protease inhibitor cocktail set (Calbiochem), PMSF 10ul/ml (from 0.1M stock, Sigma)

6.2. Protein quantification

Protein extracts were assessed for concentration using the colorimetric Pierce™ BCA Protein Assay Kit (ThermoFisher). To analyze the samples, protein extracts from muscles were diluted 1:20 and protein extracts from spinal cords 1:5. Twenty-five microliters of the resulting solutions were examined in duplicates. The working reagent (WR) was prepared by combining reagents A and B in a 50:1 ratio according to the manufacturer's instructions. Protein extracts and BSA standards were loaded in a 96-well flat plate, along with 200 µL of WR, and incubated at 37°C for 30 minutes. The absorbance at 562 nm was measured using a microplate absorbance reader (Spark, TECAN). The concentration of the sample was extrapolated from the standard curve generated.

6.3. Soluble Klotho ELISA

KL concentrations in serum, gastrocnemius muscle, and spinal cord samples were detected using an ELISA kit (27601, IBL) for mouse-soluble KL with an antibody against the KL1 domain. Manufacturer's instructions were followed after loading 100 µL of the samples on the pre-coated plate. In summary, standards were prepared in serial two-fold dilutions of recombinant mouse soluble KL in EIA buffer, ranging from 5000 to 19.5 pg/mL. Prepared test samples and standards were added to their respective wells. Following an hour of incubation at 37°C, a washing step was performed using the prepared wash buffer. Labeled antibody was added to the wells, and after a second 30 min incubation at 2-8°C, the plate was washed again. The chromogen (TMB solution) was introduced into the wells, followed by incubation in the dark for 30 min at room temperature.

The reaction was stopped with the Stop solution and optical density was determined by measuring the standard and test samples against the test sample blank, using a wavelength of 450 nm in the microplate reader (Spark, TECAN). Sample concentrations were inferred from the standard curve and, for the gastrocnemius and spinal cord samples, normalized based on the total protein loaded in each well.

7. RNA sequencing

7.1. RNA extraction

RNA seq analysis was conducted on fresh frozen samples of gastrocnemius muscles and spinal cords obtained from 16-week-old female mice. These mice belonged to four groups: WT Null, WT sKL, SOD1^{G93A} Null, and SOD1^{G93A} sKL, which had been intravenously administered MyoAAV vectors at 6 weeks of age. Gastrocnemius muscles were rapidly frozen in liquid nitrogen and then ground into powder. A stainless-steel bead was added to each pulverized sample and was lysed with 1 mL of QIAzol Lysis Reagent (QIAGEN) per 100 mg of tissue using the TissueLyser LT (QIAGEN). After centrifugation at 12,000g for 10 minutes at 4°C, the supernatant was carefully transferred to a new tube, and 0.2 mL of chloroform (Panreac) per 1 mL of QIAzol Lysis Reagent was added and vigorously vortexed. The homogenate underwent a 2–3 min incubation at room temperature and was then centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous phase was transferred to gDNA eliminator mini spin columns from the RNeasy Plus Mini Kit (QIAGEN). For spinal cord samples, 600 µL of the provided RLT buffer in the kit replaced QIAzol, and the lysate was obtained using the TissueLyser LT. Following 3-minute centrifugation at maximum speed, the supernatant was carefully transferred to a gDNA eliminator spin column. The RNA extraction process followed the RNeasy Plus Mini Kit instructions, eluting the RNA in 30 µL for spinal cord samples and 50 µL for muscle samples. RNA quantity was assessed using NanoDrop (Thermo Scientific) for muscle samples and Qubit high-sensitivity kits (Invitrogen) for spinal cord samples. RNA quality was assessed by 2100 Bioanalyzer (Agilent), ensuring that all RNA samples had a RIN>7.5, indicative of non-degraded RNA appropriate for RNAseq studies.

7.2. RNA library preparation, sequencing, mapping, and analysis

Next-generation sequencing (NGS) technologies for bulk whole-transcriptome profiling offer a comprehensive readout of transcript abundance. RNA sequencing was performed at the IRB Barcelona Functional Genomics Core Facility. In summary, total RNA libraries were created using

the TruSeq v2 kit (Illumina) to generate unstranded libraries with an average fragment size of 150 bp. The resulting libraries underwent 150-bp paired-end sequencing on an Illumina HiSeq 2000 or 2500 machine. Reads included one extra cycle for phasing purposes (151 nt).

Bioinformatics analyses consisted of several steps and were conducted at the IRB Biostatistics/bioinformatics Core Facility. Paired-end reads were aligned with STAR to the *Mus musculus* genome (Dobin et al., 2013), gene expression levels were quantified using the R package Rsubread (Liao et al., 2019) and data was transformed with variance-stabilizing transformation (VST) for exploration purposes. DESeq2 was used for differential expression analysis with independent filtering of features applied (Love et al., 2014), and multiple contrasts were adjusted with Benjamini-Hochberg FDR (Benjamini & Hochberg, 1995). For enrichment analysis, ROAST-GSA was used, a hybrid of methods that combines the statistical inference based on limma (Ritchie et al., 2015), rotations from ROAST (Wu et al., 2010), and the re-standardized MaxMean statistic from GSA (Efron & Tibshirani, 2007). To annotate genes according to GO terms labels and KEGG pathways the Bioconductors' R package "org.Mm.eg.db" was used (Carlson M.: Genome wide annotation for Mouse), and GO slim terms downloaded from the GO project webpage (geneontology.org). All these analyses were performed on DESeq2's rlog transformed counts (Love et al., 2014). To compare RNAseq and proteomic data, hypergeometric tests for functional enrichment were performed with the top/bottom differentially expressed genes/proteins, filtering them by a p-value lower than 0.1.

8. Proteomic analysis

8.1. Protein extraction

The same mice as in the RNAseq studies were employed, using the contralateral hindlimb gastrocnemius muscle or the other hemisphere of the spinal cord. A sufficient excess of lysis buffer over the sample was ensured. Samples were kept on ice to avoid overheating and were subjected to sonication at 40Hz with an ultrasonic processor (Vibra-cell™, Sonics & Materials Inc). Five cycles of sonication for 30s were alternated with cycles of cooling on ice for 30s. Subsequently, the samples were incubated at 95°C for 5 minutes with optional soft shaking. After incubation, a second round of five sonication-ice cycles was repeated. The samples were then centrifuged at 16,000g and 13°C for 20 min, and the resulting supernatant was carefully transferred to a new tube with low protein binding properties (1.5 ml).

Lysis buffer: 2% w/v SDS (USB) and 0.1M dithiothreitol (DTT, Pierce TM, ThermoFisher) in 100 mM Tris (Sigma)/HCl (Panreac), pH=7.6.

8.2. Mass spectrometry/Proteomics and data analysis

Mass spectrometry and proteomics analyses were conducted at the IRB Barcelona Mass Spectrometry and Proteomics Core Facility. Total protein content was quantified using Pierce™ 660 nm Protein Assay Reagent following the manufacturer's guidelines. FASP digestion of samples was performed with trypsin (1 µg/µL, Promega), followed by cleanup using C18 tips (PolyLC) (Wiśniewski et al., 2009). Tryptic samples were quantified at the peptide level using Pierce™ Quantitative Peptide Assays (ThermoFisher) and subjected to nanoLC-MS/MS analysis. The nanoLC-MS/MS setup included the Evosep One chromatograph (EV-1000, Evosep) with the Evotip trap column and EasySpray for coupling to the Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific). Data were acquired using a Data Independent Acquisition (DIA), library-free, label-free strategy. Random injection of samples was performed to mitigate carryover, machine drift, and run order effects.

Differential abundance analysis was carried out using limma, employing a label-free quantification approach with six distinct contrasts: SOD1 Null vs SOD1 sKL, SOD1 Null vs WT sKL, SOD1 Null vs WT Null, SOD1 sKL vs WT sKL, SOD1 sKL vs WT Null, and WT Null vs WT sKL. For protein quantification, we employed MaxLFQ (J. Cox et al., 2014) computed from normalized precursor quantities provided by DIANN using the SwissProt Mouse database (Demichev et al., 2020) and the lmFit function for model fitting from the limma package (Ritchie et al., 2015). Standard cutoffs for fold change ($|FC| > 1.5$) and adjusted p-value ($p_{adj} < 0.05$) were applied to identify significant proteins using the Benjamini & Hochberg (1995) FDR method. Data preprocessing involved excluding protein groups identified solely by a single unique peptide. Furthermore, proteins with no missing values in at least one condition were retained, and "multiple-member" protein groups redundantly represented by others in the DIA-NN output were eliminated.

9. Statistical analysis

All experiments were performed by researchers blinded to genotype when possible, and to treatment received by each mouse group. Animals were assigned randomly to groups to ensure that littermates or mice from the same cage were not grouped.

Sample sizes were determined based on prior observations in our laboratory, considering that almost all animals, regardless of their intended experimental use, were subjected to electrophysiological and behavioral testing. Statistical analysis and graphical representations were performed using GraphPad Prism version 9 (GraphPad Software) and R software.

Shapiro-Wilk normality tests were applied to verify the normal distribution of the data. Comparisons between two groups were performed with a two-tailed unpaired Student's t-test. One-way analysis of variance (ANOVA) for comparisons of one variable over all groups. Two-way ANOVA was performed when the effect of the dependent variable was tested on the genotype of the animals and the treatment. Following ANOVA, Tukey's or Sidak's multiple comparisons post-hoc tests were performed to compare multiple groups and identify significant differences between specific pairs of groups. Data are presented as mean $\pm$ standard error of the mean (SEM), and statistical significance is established at p-values ≤ 0.05 unless stated otherwise. Significant outliers were detected by Grubb's test (extreme studentized deviate) and removed from the analysis. The statistics used in RNA-seq and proteomic studies are described in their respective sections. Mantel-Cox (log-rank) test was used for assessment of the clinical disease onset, clasping onset, and survival analysis.

REFERENCES

- Ababneh, N. A., Scaber, J., Flynn, R., Douglas, A., Barbagallo, P., Candalija, A., Turner, M. R., Sims, D., Dafinca, R., Cowley, S. A., & Talbot, K. (2020). Correction of amyotrophic lateral sclerosis related phenotypes in induced pluripotent stem cell-derived motor neurons carrying a hexanucleotide expansion mutation in C9orf72 by CRISPR/Cas9 genome editing using homology-directed repair. *Human Molecular Genetics*, 29(13), 2200–2217. <https://doi.org/10.1093/hmg/ddaa106>
- Abe, K., Aoki, M., Tsuji, S., Itoyama, Y., Sobue, G., Togo, M., Hamada, C., Tanaka, M., Akimoto, M., Nakamura, K., Takahashi, F., Kondo, K., Yoshino, H., Abe, K., Aoki, M., Tsuji, S., Itoyama, Y., Sobue, G., Togo, M., ... Yoshino, H. (2017). Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *The Lancet Neurology*, 16(7), 505–512. [https://doi.org/10.1016/S1474-4422\(17\)30115-1](https://doi.org/10.1016/S1474-4422(17)30115-1)
- Abraham, C. R., Mullen, P. C., Tucker-Zhou, T., Chen, C. D., & Zeldich, E. (2016). Klotho Is a Neuroprotective and Cognition-Enhancing Protein. In *Vitamins and Hormones* (1st ed., Vol. 101). Elsevier Inc. <https://doi.org/10.1016/bs.vh.2016.02.004>
- Acevedo, J. M., Hoermann, B., Schlimbach, T., & Teleman, A. A. (2018). Changes in global translation elongation or initiation rates shape the proteome via the Kozak sequence. *Scientific Reports*, 8(1), 4018. <https://doi.org/10.1038/s41598-018-22330-9>
- Acsadi, G., Anguelov, R. A., Yang, H., Toth, G., Thomas, R., Jani, A., Wang, Y., Ianakova, E., Mohammad, S., Lewis, R. A., & Shy, M. E. (2002). Increased survival and function of SOD1 mice after glial cell-derived neurotrophic factor gene therapy. *Human Gene Therapy*, 13(9), 1047–1059. <https://doi.org/10.1089/104303402753812458>
- Ahrens, H. E., Huettemeister, J., Schmidt, M., Kaether, C., & von Maltzahn, J. (2018). Klotho expression is a prerequisite for proper muscle stem cell function and regeneration of skeletal muscle. *Skeletal Muscle*, 8(1), 1–14. <https://doi.org/10.1186/s13395-018-0166-x>
- Akintola, A. A., & van Heemst, D. (2015). Insulin, Aging, and the Brain: Mechanisms and Implications. *Frontiers in Endocrinology*, 6. <https://doi.org/10.3389/fendo.2015.00013>
- Alexander, G. M., Erwin, K. L., Byers, N., Deitch, J. S., Augelli, B. J., Blankenhorn, E. P., & Heiman-Patterson, T. D. (2004). Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS. *Molecular Brain Research*, 130(1–2), 7–15. <https://doi.org/10.1016/j.molbrainres.2004.07.002>
- Alexianu, M. E., Ho, B.-K., Mohamed, A. H., La Bella, V., Smith, R. G., & Appel, S. H. (1994). The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis. *Annals of Neurology*, 36(6), 846–858. <https://doi.org/10.1002/ana.410360608>
- Ali, M., Sung, Y. J., Wang, F., Fernández, M. V., Morris, J. C., Fagan, A. M., Blennow, K., Zetterberg, H., Heslegrave, A., Johansson, P. M., Svensson, J., Nellgård, B., Lleó, A., Alcolea, D., Clarimon, J., Rami, L., Molinuevo, J. L., Suárez-Calvet, M., Morenas-Rodríguez, E., ... Cruchaga, C. (2022). Leveraging large multi-center cohorts of Alzheimer disease endophenotypes to understand the role of Klotho heterozygosity on disease risk. *PLOS ONE*, 17(5), e0267298. <https://doi.org/10.1371/journal.pone.0267298>
- Allen, M. J., Lacroix, J. J., Ramachandran, S., Capone, R., Whitlock, J. L., Ghadge, G. D., Arnsdorf, M. F., Roos, R. P., & Lal, R. (2012). Mutant SOD1 forms ion channel: implications for ALS pathophysiology. *Neurobiology of Disease*, 45(3), 831–838. <https://doi.org/10.1016/j.nbd.2011.08.031>
- Allodi, I., Comley, L., Nichterwitz, S., Nizzardo, M., Simone, C., Benitez, J. A., Cao, M., Corti, S., & Hedlund, E. (2016). Differential neuronal vulnerability identifies IGF-2 as a protective factor in ALS. *Scientific Reports*, 6(February), 1–14. <https://doi.org/10.1038/srep25960>
- Almilaji, A., Munoz, C., Pakladok, T., Alesutan, I., Feger, M., Föller, M., Lang, U. E., Shumilina, E., & Lang, F. (2013). Klotho Sensitivity of the Neuronal Excitatory Amino Acid Transporters EAAT3 and EAAT4. *PLoS ONE*, 8(7), 48–50. <https://doi.org/10.1371/journal.pone.0070988>
- Amlie-Wolf, A., Ryvkin, P., Tong, R., Dragomir, I., Suh, E., Xu, Y., Van Deerlin, V. M., Gregory, B. D., Kwong, L. K., Trojanowski, J. Q., Lee, V. M.-Y., Wang, L.-S., & Lee, E. B. (2015). Transcriptomic Changes Due to Cytoplasmic TDP-43 Expression Reveal Dysregulation of Histone Transcripts and Nuclear Chromatin. *PLOS ONE*, 10(10), e0141836. <https://doi.org/10.1371/journal.pone.0141836>
- Anamizu, Y., Kawaguchi, H., Seichi, A., Yamaguchi, S., Kawakami, E., Kanda, N., Matsubara, S., Kuro-o, M., Nabeshima, Y., Nakamura, K., & Oyanagi, K. (2005). Klotho insufficiency causes decrease of ribosomal RNA gene transcription activity, cytoplasmic RNA and rough ER in the spinal anterior horn cells. *Acta Neuropathologica*, 109(5), 457–466. <https://doi.org/10.1007/s00401-004-0971-7>
- Anguela, X. M., & High, K. A. (2019). Entering the Modern Era of Gene Therapy. *Annual Review of Medicine*, 70(1), 273–288. <https://doi.org/10.1146/annurev-med-012017-043332>

- Aoyama, K. (2000). Nitration of manganese superoxide dismutase in cerebrospinal fluids is a marker for peroxynitrite-mediated oxidative stress in neurodegenerative diseases. *ANNALS OF NEUROLOGY*, 47(4), 524.
- Ardley, H. C., Scott, G. B., Rose, S. A., Tan, N. G. S., & Robinson, P. A. (2004). UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease. *Journal of Neurochemistry*, 90(2), 379–391. <https://doi.org/10.1111/j.1471-4159.2004.02485.x>
- Arjomandnejad, M., Dasgupta, I., Flotte, T. R., & Keeler, A. M. (2023). Immunogenicity of Recombinant Adeno-Associated Virus (AAV) Vectors for Gene Transfer. In *BioDrugs* (Vol. 37, Issue 3, pp. 311–329). Adis. <https://doi.org/10.1007/s40259-023-00585-7>
- Arundine, M., & Tymianski, M. (2003). Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium*, 34(4–5), 325–337. [https://doi.org/10.1016/S0143-4160\(03\)00141-6](https://doi.org/10.1016/S0143-4160(03)00141-6)
- Ash, P. E. A., Bieniek, K. F., Gendron, T. F., Caulfield, T., Lin, W.-L., DeJesus-Hernandez, M., van Blitterswijk, M. M., Jansen-West, K., Paul, J. W., Rademakers, R., Boylan, K. B., Dickson, D. W., & Petrucelli, L. (2013). Unconventional Translation of C9ORF72 GGGGCC Expansion Generates Insoluble Polypeptides Specific to c9FTD/ALS. *Neuron*, 77(4), 639–646. <https://doi.org/10.1016/j.neuron.2013.02.004>
- Atkin, J. D., Farg, M. A., Walker, A. K., McLean, C., Tomas, D., & Horne, M. K. (2008). Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. In *Neurobiology of Disease* (Vol. 30, Issue 3, pp. 400–407). Academic Press Inc. <https://doi.org/10.1016/j.nbd.2008.02.009>
- Ayers, J. I., Fromholt, S., Sinyavskaya, O., Siemienski, Z., Rosario, A. M., Li, A., Crosby, K. W., Cruz, P. E., Dinunno, N. M., Janus, C., Ceballos-Diaz, C., Borchelt, D. R., Golde, T. E., Chakrabarty, P., & Levites, Y. (2015). Widespread and efficient transduction of spinal cord and brain following neonatal AAV injection and potential disease modifying effect in ALS mice. *Molecular Therapy*, 23(1), 53–62. <https://doi.org/10.1038/mt.2014.180>
- Azzouz, M. (2000). Increased motoneuron survival and improved neuromuscular function in transgenic ALS mice after intraspinal injection of an adeno-associated virus encoding Bcl-2. *Human Molecular Genetics*, 9(5), 803–811. <https://doi.org/10.1093/hmg/9.5.803>
- Azzouz, M., Leclerc, N., Gurney, M., Warter, J. M., Poindron, P., & Borg, J. (1997). Progressive motor neuron impairment in an animal model of familial amyotrophic lateral sclerosis. *Muscle and Nerve*, 20(1), 45–51. [https://doi.org/10.1002/\(SICI\)1097-4598\(199701\)20:1<45::AID-MUS6>3.0.CO;2-H](https://doi.org/10.1002/(SICI)1097-4598(199701)20:1<45::AID-MUS6>3.0.CO;2-H)
- Azzouz, M., Ralph, G. S., Storkebaum, E., Walmsley, L. E., Mitrophanous, K. A., Kingsman, S. M., Carmeliet, P., & Mazarakis, N. D. (2004). VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature*, 429(6990), 413–417. <https://doi.org/10.1038/nature02544>
- Baker, R. E., Dijkhuizen, P. A., Van Pelt, J., & Verhaagen, J. (1998). Growth of pyramidal, but not non-pyramidal, dendrites in long-term organotypic explants of neonatal rat neocortex chronically exposed to neurotrophin-3. *European Journal of Neuroscience*, 10(3), 1037–1044. <https://doi.org/10.1046/j.1460-9568.1998.00118.x>
- Balcerak, A., Macech-Klicka, E., Wakula, M., Tomecki, R., Goryca, K., Rydzanicz, M., Chmielarczyk, M., Szostakowska-Rodzos, M., Wisniewska, M., Lyczek, F., Helwak, A., Tollervey, D., Kudla, G., & Grzybowska, E. A. (2022). The RNA-Binding Landscape of HAX1 Protein Indicates Its Involvement in Translation and Ribosome Assembly. *Cells*, 11(19), 2943. <https://doi.org/10.3390/cells11192943>
- Baluchnejadmojarad, T., Eftekhari, S.-M., Jamali-Raeufy, N., Haghani, S., Zeinali, H., & Roghani, M. (2017). The anti-aging protein klotho alleviates injury of nigrostriatal dopaminergic pathway in 6-hydroxydopamine rat model of Parkinson's disease: Involvement of PKA/CaMKII/CREB signaling. *Experimental Gerontology*, 100, 70–76. <https://doi.org/10.1016/j.exger.2017.10.023>
- Bame, M., Pentiak, P. A., Needleman, R., & Brusilow, W. S. A. (2012). Effect of Sex on Lifespan, Disease Progression, and the Response to Methionine Sulfoximine in the SOD1 G93A Mouse Model for ALS. *Gender Medicine*, 9(6), 524–535. <https://doi.org/10.1016/j.genm.2012.10.014>
- Banerjee, P., Mehta, A. R., Nirujogi, R. S., Cooper, J., James, O. G., Nanda, J., Longden, J., Burr, K., McDade, K., Salzinger, A., Paza, E., Newton, J., Story, D., Pal, S., Smith, C., Alessi, D. R., Selvaraj, B. T., Priller, J., & Chandran, S. (2023). Cell-autonomous immune dysfunction driven by disrupted autophagy in C9orf72-ALS iPSC-derived microglia contributes to neurodegeneration. *Science Advances*, 9(16). <https://doi.org/10.1126/sciadv.abq0651>
- Banerjee, S., Zhao, Y., Sarkar, P. S., Rosenblatt, K. P., Tilton, R. G., & Choudhary, S. (2013). Klotho Ameliorates Chemically Induced Endoplasmic Reticulum (ER) Stress Signaling. *Cellular Physiology and Biochemistry*, 31(4–5), 659–672. <https://doi.org/10.1159/000350085>

- Bankole, O., Scambi, I., Parrella, E., Muccilli, M., Bonafede, R., Turano, E., Pizzi, M., & Mariotti, R. (2022). Beneficial and Sexually Dimorphic Response to Combined HDAC Inhibitor Valproate and AMPK/SIRT1 Pathway Activator Resveratrol in the Treatment of ALS Mice. *International Journal of Molecular Sciences*, 23(3), 1047. <https://doi.org/10.3390/ijms23031047>
- Beal, M. F. (1997). Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Annals of Neurology*, 42(4), 644.
- Becker, L. A., Huang, B., Bieri, G., Ma, R., Knowles, D. A., Jafar-Nejad, P., Messing, J., Kim, H. J., Soriano, A., Auburger, G., Pulst, S. M., Taylor, J. P., Rigo, F., & Gitler, A. D. (2017). Therapeutic reduction of ataxin-2 extends lifespan and reduces pathology in TDP-43 mice. *Nature*, 544(7650), 367–371. <https://doi.org/10.1038/nature22038>
- Beers, D. R., & Appel, S. H. (2019). Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies. *The Lancet Neurology*, 18(2), 211–220. [https://doi.org/10.1016/S1474-4422\(18\)30394-6](https://doi.org/10.1016/S1474-4422(18)30394-6)
- Beers, D. R., Henkel, J. S., Xiao, Q., Zhao, W., Wang, J., Yen, A. A., Siklos, L., McKercher, S. R., & Appel, S. H. (2006). Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences*, 103(43), 16021–16026. <https://doi.org/10.1073/pnas.0607423103>
- Behringer, V., Stevens, J. M. G., Deschner, T., Sonnweber, R., & Hohmann, G. (2018). Aging and sex affect soluble alpha klotho levels in bonobos and chimpanzees. *Frontiers in Zoology*, 15(1), 35. <https://doi.org/10.1186/s12983-018-0282-9>
- Beijer, D., Kim, H. J., Guo, L., O'Donovan, K., Mademan, I., Deconinck, T., Van Schil, K., Fare, C. M., Drake, L. E., Ford, A. F., Kochański, A., Kabzińska, D., Dubuisson, N., Van den Bergh, P., Voermans, N. C., Lemmers, R. J. L. F., van der Maarel, S. M., Bonner, D., Sampson, J. B., ... Baets, J. (2021). Characterization of HNRNPA1 mutations defines diversity in pathogenic mechanisms and clinical presentation. *JCI Insight*, 6(14). <https://doi.org/10.1172/jci.insight.148363>
- Belloy, M. E., Eger, S. J., Le Guen, Y., Napolioni, V., Deters, K. D., Yang, H.-S., Scelsi, M. A., Porter, T., James, S.-N., Wong, A., Schott, J. M., Sperling, R. A., Laws, S. M., Mormino, E. C., He, Z., Han, S. S., Altmann, A., & Greicius, M. D. (2021). KL*VS heterozygosity reduces brain amyloid in asymptomatic at-risk APOE*4 carriers. *Neurobiology of Aging*, 101, 123–129. <https://doi.org/10.1016/j.neurobiolaging.2021.01.008>
- Benatar, M. (2007). Lost in translation: Treatment trials in the SOD1 mouse and in human ALS. *Neurobiology of Disease*, 26(1), 1–13. <https://doi.org/10.1016/j.nbd.2006.12.015>
- Benatar, M., Wu, J., & Turner, M. R. (2023). Neurofilament light chain in drug development for amyotrophic lateral sclerosis: a critical appraisal. *Brain*, 146(7), 2711–2716. <https://doi.org/10.1093/brain/awac394>
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, 57(1), 289–300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Benkler, C., Barhum, Y., Ben-Zur, T., & Offen, D. (2016). Multifactorial Gene Therapy Enhancing the Glutamate Uptake System and Reducing Oxidative Stress Delays Symptom Onset and Prolongs Survival in the SOD1-G93A ALS Mouse Model. *Journal of Molecular Neuroscience*, 58(1), 46–58. <https://doi.org/10.1007/s12031-015-0695-2>
- Bensimon, G., Lacomblez, L., & Meininger, V. (1994). A Controlled Trial of Riluzole in Amyotrophic Lateral Sclerosis. *New England Journal of Medicine*, 330(9), 585–591. <https://doi.org/10.1056/NEJM199403033300901>
- Bernal, A. F., Mota, N., Pamplona, R., Area-Gomez, E., & Portero-Otin, M. (2023). Hakuna MAM-Tata: Investigating the role of mitochondrial-associated membranes in ALS. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1869(6), 166716. <https://doi.org/10.1016/j.bbadis.2023.166716>
- Bersano, E., Sarnelli, M. F., Solara, V., Iazzolino, B., Peotta, L., De Marchi, F., Facchin, A., Moglia, C., Canosa, A., Calvo, A., Chiò, A., & Mazzini, L. (2020). Decline of cognitive and behavioral functions in amyotrophic lateral sclerosis: a longitudinal study. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, 21(5–6), 373–379. <https://doi.org/10.1080/21678421.2020.1771732>
- Bian, A., Neyra, J. A., Zhan, M., & Hu, M. C. (2015). Klotho, stem cells, and aging. *Clinical Interventions in Aging*, 10, 1233–1243. <https://doi.org/10.2147/CIA.S84978>
- Biferi, M. G., Cohen-Tannoudji, M., Cappelletto, A., Giroux, B., Roda, M., Astord, S., Marais, T., Bos, C., Voit, T., Ferry, A., & Barkats, M. (2017). A New AAV10-U7-Mediated Gene Therapy Prolongs Survival and

- Restores Function in an ALS Mouse Model. *Molecular Therapy*, 25(9), 2038–2052. <https://doi.org/10.1016/j.ymthe.2017.05.017>
- Bilslund, L. G., Sahai, E., Kelly, G., Golding, M., Greensmith, L., & Schiavo, G. (2010). Deficits in axonal transport precede ALS symptoms in vivo. *Proceedings of the National Academy of Sciences*, 107(47), 20523–20528. <https://doi.org/10.1073/pnas.1006869107>
- Bogdanov, M., Brown, R. H., Matson, W., Smart, R., Hayden, D., O'Donnell, H., Flint Beal, M., & Cudkovic, M. (2000). Increased oxidative damage to DNA in ALS patients. *Free Radical Biology and Medicine*, 29(7), 652–658. [https://doi.org/10.1016/S0891-5849\(00\)00349-X](https://doi.org/10.1016/S0891-5849(00)00349-X)
- Boill  e, S., Yamanaka, K., Lobsiger, C. S., Copeland, N. G., Jenkins, N. A., Kassiotis, G., Kollias, G., & Cleveland, D. W. (2006). Onset and Progression in Inherited ALS Determined by Motor Neurons and Microglia. *Science*, 312(5778), 1389–1392. <https://doi.org/10.1126/science.1123511>
- Bordet, T., Lesbordes, J.-C., Rouhani, S., Castelnuovo-Ptakhine, L., Schmalbruch, H., Haase, G., & Kahn, A. (2001). Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice. In *Human Molecular Genetics* (Vol. 10).
- Borel, F., Gernoux, G., Cardozo, B., Metterville, J. P., Cabrera, G. T., Song, L., Su, Q., Gao, G. P., Elmallah, M. K., Jr, R. H. B., & Mueller, C. (2016). *Therapeutic rAAVrh10 Mediated SOD1 Silencing in Adult SOD1 G93A Mice and Nonhuman Primates*. 27(1). <https://doi.org/10.1089/hum.2015.122>
- Borel, F., Gernoux, G., Sun, H., Stock, R., Blackwood, M., Brown, R. H., & Mueller, C. (2018). Safe and effective superoxide dismutase 1 silencing using artificial microRNA in macaques. *Science Translational Medicine*, 10(465). <https://doi.org/10.1126/scitranslmed.aau6414>
- Borthwick, G. M., Johnson, M. A., Ince, P. G., Shaw, P. J., & Turnbull, D. M. (1999). Mitochondrial enzyme activity in amyotrophic lateral sclerosis: Implications for the role of mitochondria in neuronal cell death. *Annals of Neurology*, 46(5), 787–790. [https://doi.org/10.1002/1531-8249\(199911\)46:5<787::AID-ANA17>3.0.CO;2-8](https://doi.org/10.1002/1531-8249(199911)46:5<787::AID-ANA17>3.0.CO;2-8)
- Braak, H., Bretschneider, J., Ludolph, A. C., Lee, V. M., John, Q., & Tredici, K. Del. (2014). Amyotrophic lateral sclerosis - a model of corticofugal axonal spread. *Nature Reviews Neurology*, 9(12), 708–714. <https://doi.org/10.1038/nrneurol.2013.221>
- Bravo-Hernandez, M., Tadokoro, T., Navarro, M. R., Platoshyn, O., Kobayashi, Y., Marsala, S., Miyahara, A., Juhas, S., Juhasova, J., Skaln  kova, H., Tomori, Z., Vanicky, I., Studenovska, H., Proks, V., Chen, P. X., Govea-Perez, N., Ditsworth, D., Ciacchi, J. D., Gao, S., ... Marsala, M. (2020). Spinal subpial delivery of AAV9 enables widespread gene silencing and blocks motoneuron degeneration in ALS. *Nature Medicine*, 26(1), 118–130. <https://doi.org/10.1038/s41591-019-0674-1>
- Brenner, M., Kisseberth, W., Su, Y., Besnard, F., & Messing, A. (1994). GFAP promoter directs astrocyte-specific expression in transgenic mice. *The Journal of Neuroscience*, 14(3), 1030–1037. <https://doi.org/10.1523/JNEUROSCI.14-03-01030.1994>
- Bristol, L. A., & Rothstein, J. D. (1996). Glutamate transporter gene expression in amyotrophic lateral sclerosis motor cortex. *Annals of Neurology*, 39(5), 676–679. <https://doi.org/10.1002/ana.410390519>
- Brobey, R. K., German, D., Sonsalla, P. K., Gurnani, P., Pastor, J., Hsieh, C. C., Papaconstantinou, J., Foster, P. P., Kuro-O, M., & Rosenblatt, K. P. (2015). Klotho protects dopaminergic neuron oxidant-induced degeneration by modulating ASK1 and p38 MAPK signaling pathways. *PLoS ONE*, 10(10), 1–15. <https://doi.org/10.1371/journal.pone.0139914>
- Brown, A.-L., Wilkins, O. G., Keuss, M. J., Hill, S. E., Zanovello, M., Lee, W. C., Bampton, A., Lee, F. C. Y., Masino, L., Qi, Y. A., Bryce-Smith, S., Gatt, A., Hallegger, M., Fagegaltier, D., Phatnani, H., Phatnani, H., Kwan, J., Sareen, D., Broach, J. R., ... Fratta, P. (2022). TDP-43 loss and ALS-risk SNPs drive mis-splicing and depletion of UNC13A. *Nature*, 603(7899), 131–137. <https://doi.org/10.1038/s41586-022-04436-3>
- Brown, R. H., & Al-Chalabi, A. (2017). Amyotrophic Lateral Sclerosis. *New England Journal of Medicine*, 377(2), 162–172. <https://doi.org/10.1056/NEJMr1603471>
- Brunet, N., Tarabal, O., Esquerda, J. E., & Calder  , J. (2009). Excitotoxic motoneuron degeneration induced by glutamate receptor agonists and mitochondrial toxins in organotypic cultures of chick embryo spinal cord. *The Journal of Comparative Neurology*, 516(4), 277–290. <https://doi.org/10.1002/cne.22118>
- Bruneteau, G., Bauch  , S., Gonzalez de Aguilar, J. L., Brochier, G., Mandjee, N., Tanguy, M.-L., Hussain, G., Behin, A., Khiami, F., Soriali, E., Hell-Remy, C., Salachas, F., Pradat, P.-F., Lacomblez, L., Nicole, S., Fontaine, B., Fardeau, M., Loeffler, J.-P., Meininger, V., ... Hanta  , D. (2015). Endplate denervation correlates with Nogo-A muscle expression in amyotrophic lateral sclerosis patients. *Annals of Clinical and Translational Neurology*, 2(4), 362–372. <https://doi.org/10.1002/acn3.179>

- Bucher, T., Colle, M. A., Wakeling, E., Dubreil, L., Fyfe, J., Briot-Nivard, D., Maquigneau, M., Raoul, S., Cherel, Y., Astord, S., Duque, S., Marais, T., Voit, T., Moullier, P., Barkats, M., & Joussemet, B. (2013). ScAAV9 intracisternal delivery results in efficient gene transfer to the central nervous system of a feline model of motor neuron disease. *Human Gene Therapy*, 24(7), 670–682. <https://doi.org/10.1089/hum.2012.218>
- Bulcha, J. T., Wang, Y., Ma, H., Tai, P. W. L., & Gao, G. (2021). Viral vector platforms within the gene therapy landscape. *Signal Transduction and Targeted Therapy*, 6(1), 53. <https://doi.org/10.1038/s41392-021-00487-6>
- Burk, K., & Pasterkamp, R. J. (2019). Disrupted neuronal trafficking in amyotrophic lateral sclerosis. *Acta Neuropathologica*, 137(6), 859–877. <https://doi.org/10.1007/s00401-019-01964-7>
- Burkhardt, M. F., Martinez, F. J., Wright, S., Ramos, C., Volfson, D., Mason, M., Garnes, J., Dang, V., Lievers, J., Shoukat-Mumtaz, U., Martinez, R., Gai, H., Blake, R., Vaisberg, E., Grskovic, M., Johnson, C., Irion, S., Bright, J., Cooper, B., ... Javaherian, A. (2013). A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells. *Molecular and Cellular Neuroscience*, 56, 355–364. <https://doi.org/10.1016/j.mcn.2013.07.007>
- Calandra, T., Froidevaux, C., Martin, C., & Roger, T. (2003). Macrophage Migration Inhibitory Factor and Host Innate Immune Defenses against Bacterial Sepsis. *The Journal of Infectious Diseases*, 187(s2), S385–S390. <https://doi.org/10.1086/374752>
- Calvo, A. C., Cibreiro, G. A., Merino, P. T., Roy, J. F., Galiana, A., Rufián, A. J., Cano, J. M., Martín, M. A., Moreno, L., Larrodé, P., Vázquez, P. C., Galán, L., Mora, J., Muñoz-Blanco, J. L., Muñoz, M. J., Zaragoza, P., Pegoraro, E., Sorarù, G., Mora, M., ... Redondo, A. G. (2019). Collagen XIX Alpha 1 Improves Prognosis in Amyotrophic Lateral Sclerosis. *Aging and Disease*, 10(2), 278. <https://doi.org/10.14336/AD.2018.0917>
- Calvo, A. C., Manzano, R., Atencia-Cibreiro, G., Oliván, S., Muñoz, M. J., Zaragoza, P., Cordero-Vázquez, P., Esteban-Pérez, J., García-Redondo, A., & Osta, R. (2012). Genetic biomarkers for ALS disease in transgenic SOD1 G93A mice. *PLoS ONE*, 7(3). <https://doi.org/10.1371/journal.pone.0032632>
- Cao, Y., Kumar, R. M., Penn, B. H., Berkes, C. A., Kooperberg, C., Boyer, L. A., Young, R. A., & Tapscott, S. J. (2006). Global and gene-specific analyses show distinct roles for Myod and Myog at a common set of promoters. *The EMBO Journal*, 25(3), 502–511. <https://doi.org/10.1038/sj.emboj.7600958>
- Carriedo, S. G., Sensi, S. L., Yin, H. Z., & Weiss, J. H. (2000). AMPA Exposures Induce Mitochondrial Ca²⁺ Overload and ROS Generation in Spinal Motor Neurons *In Vitro*. *The Journal of Neuroscience*, 20(1), 240–250. <https://doi.org/10.1523/JNEUROSCI.20-01-00240.2000>
- Carriedo, S. G., Yin, H. Z., & Weiss, J. H. (1996). Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 16(13), 4069–4079. <https://doi.org/10.1523/JNEUROSCI.16-13-04069.1996>
- Casci, I., Krishnamurthy, K., Kour, S., Tripathy, V., Ramesh, N., Anderson, E. N., Marrone, L., Grant, R. A., Oliver, S., Gochenaur, L., Patel, K., Sternecker, J., Gleixner, A. M., Donnelly, C. J., Ruepp, M.-D., Sini, A. M., Zuccaro, E., Pennuto, M., Pasinelli, P., & Pandey, U. B. (2019). Muscleblind acts as a modifier of FUS toxicity by modulating stress granule dynamics and SMN localization. *Nature Communications*, 10(1), 5583. <https://doi.org/10.1038/s41467-019-13383-z>
- Cashman, N. R., Durham, H. D., Blusztajn, J. K., Oda, K., Tabira, T., Shaw, I. T., Dahrouge, S., & Antel, J. P. (1992). Neuroblastoma × spinal cord (NSC) hybrid cell lines resemble developing motor neurons. *Developmental Dynamics*, 194(3), 209–221. <https://doi.org/10.1002/aja.1001940306>
- Castner, S. A., Gupta, S., Wang, D., Moreno, A. J., Park, C., Chen, C., Poon, Y., Groen, A., Greenberg, K., David, N., Boone, T., Baxter, M. G., Williams, G. V., & Dubal, D. B. (2023). Longevity factor klotho enhances cognition in aged nonhuman primates. *Nature Aging*, 3(8), 931–937. <https://doi.org/10.1038/s43587-023-00441-x>
- Challis, R. C., Ravindra Kumar, S., Chan, K. Y., Challis, C., Beadle, K., Jang, M. J., Kim, H. M., Rajendran, P. S., Tompkins, J. D., Shivkumar, K., Deverman, B. E., & Gradinaru, V. (2019). Systemic AAV vectors for widespread and targeted gene delivery in rodents. *Nature Protocols*, 14(2), 379–414. <https://doi.org/10.1038/s41596-018-0097-3>
- Chang, Y., Kong, Q., Shan, X., Tian, G., Ilieva, H., Cleveland, D. W., Rothstein, J. D., Borchelt, D. R., Wong, P. C., & Lin, C. L. G. (2008). Messenger RNA oxidation occurs early in disease pathogenesis and promotes motor neuron degeneration in ALS. *PLoS ONE*, 3(8). <https://doi.org/10.1371/journal.pone.0002849>
- Chen, C., Ding, X., Akram, N., Xue, S., & Luo, S.-Z. (2019). Fused in Sarcoma: Properties, Self-Assembly and Correlation with Neurodegenerative Diseases. *Molecules (Basel, Switzerland)*, 24(8). <https://doi.org/10.3390/molecules24081622>

- Chen, C. Di, Li, H., Liang, J., Hixson, K., Zeldich, E., & Abraham, C. R. (2014). The Anti-Aging and Tumor Suppressor Protein Klotho Enhances Differentiation of a Human Oligodendrocytic Hybrid Cell Line. *Journal of Molecular Neuroscience*, 55(1), 76–90. <https://doi.org/10.1007/s12031-014-0336-1>
- Chen, C. Di, Sloane, J. A., Li, H., Aytan, N., Giannaris, E. L., Zeldich, E., Hinman, J. D., Dedeoglu, A., Rosene, D. L., Bansal, R., Luebke, J. I., Kuro-o, M., & Abraham, C. R. (2013). The antiaging protein klotho enhances oligodendrocyte maturation and myelination of the CNS. *Journal of Neuroscience*, 33(5), 1927–1939. <https://doi.org/10.1523/JNEUROSCI.2080-12.2013>
- Chen, G., Liu, Y., Goetz, R., Fu, L., Jayaraman, S., Hu, M.-C., Moe, O. W., Liang, G., Li, X., & Mohammadi, M. (2018). α -Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. *Nature*, 553(7689), 461–466. <https://doi.org/10.1038/nature25451>
- Chen, X., He, X., Yang, Y.-Y., & Wang, Y. (2022). Amyotrophic Lateral Sclerosis-Associated Mutants of SOD1 Modulate miRNA Biogenesis through Aberrant Interactions with Exportin 5. *ACS Chemical Biology*, 17(12), 3450–3457. <https://doi.org/10.1021/acscchembio.2c00591>
- Chen, Y. A., Kankel, M. W., Hana, S., Lau, S. K., Zavodszky, M. I., McKissick, O., Mastrangelo, N., Dion, J., Wang, B., Ferretti, D., Koske, D., Lehman, S., Koszka, K., McLaughlin, H., Liu, M., Marshall, E., Fabian, A. J., Cullen, P., Marsh, G., ... Lo, S.-C. (2023). In vivo genome editing using novel AAV-PHP variants rescues motor function deficits and extends survival in a SOD1-ALS mouse model. *Gene Therapy*, 30(5), 443–454. <https://doi.org/10.1038/s41434-022-00375-w>
- Chen, Y.-Z., Bennett, C. L., Huynh, H. M., Blair, I. P., Puls, I., Irobi, J., Dierick, I., Abel, A., Kennerson, M. L., Rabin, B. A., Nicholson, G. A., Auer-Grumbach, M., Wagner, K., De Jonghe, P., Griffin, J. W., Fischbeck, K. H., Timmerman, V., Cornblath, D. R., & Chance, P. F. (2004). DNA/RNA Helicase Gene Mutations in a Form of Juvenile Amyotrophic Lateral Sclerosis (ALS4). *The American Journal of Human Genetics*, 74(6), 1128–1135. <https://doi.org/10.1086/421054>
- Cheng, W., Huang, J., Fu, X.-Q., Tian, W.-Y., Zeng, P.-M., Li, Y., & Luo, Z.-G. (2023). Intrathecal delivery of AAV-NDNF ameliorates disease progression of ALS mice. *Molecular Therapy*. <https://doi.org/10.1016/j.ymthe.2023.09.018>
- Chew, J., Cook, C., Gendron, T. F., Jansen-West, K., del Rosso, G., Daugherty, L. M., Castanedes-Casey, M., Kurti, A., Stankowski, J. N., Disney, M. D., Rothstein, J. D., Dickson, D. W., Fryer, J. D., Zhang, Y.-J., & Petrucelli, L. (2019). Aberrant deposition of stress granule-resident proteins linked to C9orf72-associated TDP-43 proteinopathy. *Molecular Neurodegeneration*, 14(1), 9. <https://doi.org/10.1186/s13024-019-0310-z>
- Chew, J., Gendron, T. F., Prudencio, M., Sasaguri, H., Zhang, Y.-J., Castanedes-Casey, M., Lee, C. W., Jansen-West, K., Kurti, A., Murray, M. E., Bieniek, K. F., Bauer, P. O., Whitelaw, E. C., Rousseau, L., Stankowski, J. N., Stetler, C., Daugherty, L. M., Perkerson, E. A., Desaro, P., ... Petrucelli, L. (2015). C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science*, 348(6239), 1151–1154. <https://doi.org/10.1126/science.aaa9344>
- Chew, W. L., Tabebordbar, M., Cheng, J. K. W., Mali, P., Wu, E. Y., Ng, A. H. M., Zhu, K., Wagers, A. J., & Church, G. M. (2016). A multifunctional AAV-CRISPR-Cas9 and its host response. *Nature Methods*, 13(10), 868–874. <https://doi.org/10.1038/nmeth.3993>
- Chiot, A., Roemer, S. F., Ryner, L., Bogachuk, A., Emberley, K., Brownell, D., Jimenez, G. A., Leviten, M., Woltjer, R., Dickson, D. W., Steinman, L., & Ajami, B. (2023). Elevated $\alpha 5$ integrin expression on myeloid cells in motor areas in amyotrophic lateral sclerosis is a therapeutic target. *Proceedings of the National Academy of Sciences*, 120(32). <https://doi.org/10.1073/pnas.2306731120>
- Chiu, I. M., Chen, A., Zheng, Y., Kosaras, B., Tsiftoglou, S. A., Vartanian, T. K., Brown, R. H., & Carroll, M. C. (2008). T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS. *Proceedings of the National Academy of Sciences*, 105(46), 17913–17918. <https://doi.org/10.1073/pnas.0804610105>
- Ciryam, P., Kundra, R., Morimoto, R. I., Dobson, C. M., & Vendruscolo, M. (2015). Supersaturation is a major driving force for protein aggregation in neurodegenerative diseases. *Trends in Pharmacological Sciences*, 36(2), 72–77. <https://doi.org/10.1016/j.tips.2014.12.004>
- Collard, J. F., Côté, F., & Julien, J. P. (1995). Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature*, 375(6526), 61–64. <https://doi.org/10.1038/375061a0>
- Collins, K. A., Ambrosio, F., Rogers, R. J., Lang, W., Schelbert, E. B., Davis, K. K., & Jakicic, J. M. (2023). Change in circulating klotho in response to weight loss, with and without exercise, in adults with overweight or obesity. *Frontiers in Aging*, 4. <https://doi.org/10.3389/fragi.2023.1213228>

- Comley, L., Allodi, I., Nichterwitz, S., Nizzardo, M., Simone, C., Corti, S., & Hedlund, E. (2015). Motor neurons with differential vulnerability to degeneration show distinct protein signatures in health and ALS. *Neuroscience*, 291, 216–229. <https://doi.org/10.1016/j.neuroscience.2015.02.013>
- Cooper-Knock, J., Walsh, M. J., Higginbottom, A., Robin Highley, J., Dickman, M. J., Edbauer, D., Ince, P. G., Wharton, S. B., Wilson, S. A., Kirby, J., Hautbergue, G. M., & Shaw, P. J. (2014). Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions. *Brain*, 137(7), 2040–2051. <https://doi.org/10.1093/brain/awu120>
- Corrêa, H. de L., Raab, A. T. O., Araújo, T. M., Deus, L. A., Reis, A. L., Honorato, F. S., Rodrigues-Silva, P. L., Neves, R. V. P., Brunetta, H. S., Mori, M. A. da S., Franco, O. L., & Rosa, T. dos S. (2022). A systematic review and meta-analysis demonstrating Klotho as an emerging exerkin. *Scientific Reports*, 12(1), 17587. <https://doi.org/10.1038/s41598-022-22123-1>
- Correia, J. C., Jannig, P. R., Gosztyla, M. L., Cervenka, I., Ducommun, S., Praestholm, S. M., Dumont, K., Liu, Z., Liang, Q., Edsgård, D., Emanuelsson, O., Gregorevic, P., Westerblad, H., Venckunas, T., Brazaitis, M., Kamandulis, S., Lanner, J. T., Yeo, G. W., & Ruas, J. L. (2023). Title: *Zfp697 is an RNA-binding protein that regulates skeletal muscle inflammation and regeneration*. <https://doi.org/10.1101/2023.06.12.544338>
- Cortes, C. J., Ling, S.-C., Guo, L. T., Hung, G., Tsunemi, T., Ly, L., Tokunaga, S., Lopez, E., Sopher, B. L., Bennett, C. F., Shelton, G. D., Cleveland, D. W., & La Spada, A. R. (2014). Muscle Expression of Mutant Androgen Receptor Accounts for Systemic and Motor Neuron Disease Phenotypes in Spinal and Bulbar Muscular Atrophy. *Neuron*, 82(2), 295–307. <https://doi.org/10.1016/j.neuron.2014.03.001>
- Corti, S., Donadoni, C., Ronchi, D., Bordoni, A., Fortunato, F., Santoro, D., Del Bo, R., Lucchini, V., Crugnola, V., Papadimitriou, D., Salani, S., Moggio, M., Bresolin, N., & Comi, G. P. (2009). Amyotrophic lateral sclerosis linked to a novel SOD1 mutation with muscle mitochondrial dysfunction. *Journal of the Neurological Sciences*, 276(1–2), 170–174. <https://doi.org/10.1016/j.jns.2008.09.030>
- Couthouis, J., Hart, M. P., Erion, R., King, O. D., Diaz, Z., Nakaya, T., Ibrahim, F., Kim, H.-J., Mojsilovic-Petrovic, J., Panossian, S., Kim, C. E., Frackelton, E. C., Solski, J. A., Williams, K. L., Clay-Falcone, D., Elman, L., McCluskey, L., Greene, R., Hakonarson, H., ... Gitler, A. D. (2012). Evaluating the role of the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis. *Human Molecular Genetics*, 21(13), 2899–2911. <https://doi.org/10.1093/hmg/dds116>
- Couthouis, J., Hart, M. P., Shorter, J., DeJesus-Hernandez, M., Erion, R., Oristano, R., Liu, A. X., Ramos, D., Jethava, N., Hosangadi, D., Epstein, J., Chiang, A., Diaz, Z., Nakaya, T., Ibrahim, F., Kim, H.-J., Solski, J. A., Williams, K. L., Mojsilovic-Petrovic, J., ... Gitler, A. D. (2011). A yeast functional screen predicts new candidate ALS disease genes. *Proceedings of the National Academy of Sciences*, 108(52), 20881–20890. <https://doi.org/10.1073/pnas.1109434108>
- Cox, G. A., Lutz, C. M., Yang, C.-L., Biemesderfer, D., Bronson, R. T., Fu, A., Aronson, P. S., Noebels, J. L., & Frankel, W. N. (1997). Sodium/Hydrogen Exchanger Gene Defect in Slow-Wave Epilepsy Mutant Mice. *Cell*, 91(1), 139–148. [https://doi.org/10.1016/S0092-8674\(01\)80016-7](https://doi.org/10.1016/S0092-8674(01)80016-7)
- Cox, J., Hein, M. Y., Lubner, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Molecular & Cellular Proteomics*, 13(9), 2513–2526. <https://doi.org/10.1074/mcp.M113.031591>
- Crewe, M., & Madabhushi, R. (2021). Topoisomerase-Mediated DNA Damage in Neurological Disorders. *Frontiers in Aging Neuroscience*, 13. <https://doi.org/10.3389/fnagi.2021.751742>
- Crockford, C., Newton, J., Lonergan, K., Chiwera, T., Booth, T., Chandran, S., Colville, S., Heverin, M., Mays, I., Pal, S., Pender, N., Pinto-Grau, M., Radakovic, R., Shaw, C. E., Stephenson, L., Swinger, R., Vajda, A., Al-Chalabi, A., Hardiman, O., & Abrahams, S. (2018). ALS-specific cognitive and behavior changes associated with advancing disease stage in ALS. *Neurology*, 91(15), E1370–E1380. <https://doi.org/10.1212/WNL.0000000000006317>
- Cui, W., Len, B., Liu, W., & Wang, G. (2018). Suppression of apoptosis in human umbilical vein endothelial cells (HUVECs) by klotho protein is associated with reduced endoplasmic reticulum oxidative stress and activation of the PI3K/AKT pathway. *Medical Science Monitor*, 24, 8489–8499. <https://doi.org/10.12659/MSM.911202>
- Dadon-Nachum, M., Ben-Yaacov, K., Ben-Zur, T., Barhum, Y., Yaffe, D., Perlson, E., & Offen, D. (2014). Transplanted Modified Muscle Progenitor Cells Expressing a Mixture of Neurotrophic Factors Delay Disease Onset and Enhance Survival in the SOD1 Mouse Model of ALS. *Journal of Molecular Neuroscience*, 55(3), 788–797. <https://doi.org/10.1007/s12031-014-0426-0>

- Dadon-Nachum, M., Ben-Yaacov, K., Ben-Zur, T., Barhum, Y., Yaffe, D., Perlson, E., & Offen, D. (2015). Transplanted Modified Muscle Progenitor Cells Expressing a Mixture of Neurotrophic Factors Delay Disease Onset and Enhance Survival in the SOD1 Mouse Model of ALS. *Journal of Molecular Neuroscience*, 55(3), 788–797. <https://doi.org/10.1007/s12031-014-0426-0>
- Dalton, G., An, S.-W., Al-Juboori, S. I., Nischan, N., Yoon, J., Dobrinskikh, E., Hilgemann, D. W., Xie, J., Luby-Phelps, K., Kohler, J. J., Birnbaumer, L., & Huang, C.-L. (2017). Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling. *Proceedings of the National Academy of Sciences*, 114(4), 752–757. <https://doi.org/10.1073/pnas.1620301114>
- Damiano, M., Starkov, A. A., Petri, S., Kipiani, K., Kiaei, M., Mattiazzi, M., Flint Beal, M., & Manfredi, G. (2006). Neural mitochondrial Ca²⁺ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *Journal of Neurochemistry*, 96(5), 1349–1361. <https://doi.org/10.1111/j.1471-4159.2006.03619.x>
- de Oliveira, R. M. (2006). Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway. *FEBS LETTERS*, 580(24), 5753.
- De Vos, K. J., Chapman, A. L., Tennant, M. E., Manser, C., Tudor, E. L., Lau, K.-F., Brownlees, J., Ackerley, S., Shaw, P. J., McLoughlin, D. M., Shaw, C. E., Leigh, P. N., Miller, C. C. J., & Grierson, A. J. (2007). Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. *Human Molecular Genetics*, 16(22), 2720–2728. <https://doi.org/10.1093/hmg/ddm226>
- DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., Boxer, A. L., Baker, M., Rutherford, N. J., Nicholson, A. M., Finch, N. A., Flynn, H., Adamson, J., Kouri, N., Wojtas, A., Sengdy, P., Hsiung, G.-Y. R., Karydas, A., Seeley, W. W., Josephs, K. A., Coppola, G., Geschwind, D. H., ... Rademakers, R. (2011). Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS. *Neuron*, 72(2), 245–256. <https://doi.org/10.1016/j.neuron.2011.09.011>
- Demichev, V., Messner, C. B., Vernardis, S. I., Lilley, K. S., & Ralser, M. (2020). DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nature Methods*, 17(1), 41–44. <https://doi.org/10.1038/s41592-019-0638-x>
- Dengler, R., Konstanzer, A., Küther, G., Hesse, S., Wolf, W., & Struppler, A. (1990). Amyotrophic lateral sclerosis: macro-EMG and twitch forces of single motor units. *Muscle & Nerve*, 13(6), 545–550. <https://doi.org/10.1002/mus.880130612>
- Devine, M. S., Kiernan, M. C., Heggie, S., McCombe, P. A., & Henderson, R. D. (2014). Study of motor asymmetry in ALS indicates an effect of limb dominance on onset and spread of weakness, and an important role for upper motor neurons. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, 15(7–8), 481–487. <https://doi.org/10.3109/21678421.2014.906617>
- Dewil, M., dela Cruz, V. F., Van Den Bosch, L., & Robberecht, W. (2007). Inhibition of p38 mitogen activated protein kinase activation and mutant SOD1G93A-induced motor neuron death. *Neurobiology of Disease*, 26(2), 332–341. <https://doi.org/10.1016/j.nbd.2006.12.023>
- Di Giorgio, F. P., Boulting, G. L., Bobrowicz, S., & Eggan, K. C. (2008). Human Embryonic Stem Cell-Derived Motor Neurons Are Sensitive to the Toxic Effect of Glial Cells Carrying an ALS-Causing Mutation. *Cell Stem Cell*, 3(6), 637–648. <https://doi.org/10.1016/j.stem.2008.09.017>
- Díaz-Amarilla, P., Olivera-Bravo, S., Trias, E., Cagnolini, A., Martínez-Palma, L., Cassina, P., Beckman, J., & Barbeito, L. (2011). Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences*, 108(44), 18126–18131. <https://doi.org/10.1073/pnas.1110689108>
- Dimos, J. T., Rodolfa, K. T., Niakan, K. K., Weisenthal, L. M., Mitsumoto, H., Chung, W., Croft, G. F., Saphier, G., Leibel, R., Golland, R., Wichterle, H., Henderson, C. E., & Eggan, K. (2008). Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons. *Science*, 321(5893), 1218–1221. <https://doi.org/10.1126/science.1158799>
- Dirren, E., Aebischer, J., Rochat, C., Towne, C., Schneider, B. L., & Aebischer, P. (2015). SOD1 silencing in motoneurons or glia rescues neuromuscular function in ALS mice. *Annals of Clinical and Translational Neurology*, 2(2), 167–184. <https://doi.org/10.1002/acn3.162>
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15–21. <https://doi.org/10.1093/bioinformatics/bts635>
- Doble, A. (1996). The pharmacology and mechanism of action of riluzole. *Neurology*, 47(Issue 6, Supplement 4), 233S–241S. https://doi.org/10.1212/WNL.47.6_Suppl_4.233S

- Dobrowolny, G., Aucello, M., Rizzuto, E., Beccafico, S., Mammucari, C., Boncompagni, S., Belia, S., Wannenes, F., Nicoletti, C., Del Prete, Z., Rosenthal, N., Molinaro, M., Protasi, F., Fanò, G., Sandri, M., & Musarò, A. (2008). Skeletal Muscle Is a Primary Target of SOD1G93A-Mediated Toxicity. *Cell Metabolism*, 8(5), 425–436. <https://doi.org/10.1016/j.cmet.2008.09.002>
- Dobrowolny, G., Martone, J., Lepore, E., Casola, I., Petrucci, A., Inghilleri, M., Morlando, M., Colantoni, A., Scicchitano, B. M., Calvo, A., Bisogni, G., Chiò, A., Sabatelli, M., Bozzoni, I., & Musarò, A. (2021). A longitudinal study defined circulating microRNAs as reliable biomarkers for disease prognosis and progression in ALS human patients. *Cell Death Discovery*, 7(1), 4. <https://doi.org/10.1038/s41420-020-00397-6>
- Dodge, J. C., Haidet, A. M., Yang, W., Passini, M. A., Hester, M., Clarke, J., Roskelley, E. M., Treleaven, C. M., Rizo, L., Martin, H., Kim, S. H., Kaspar, R., Taksir, T. V., Griffiths, D. A., Cheng, S. H., Shihabuddin, L. S., & Kaspar, B. K. (2008). Delivery of AAV-IGF-1 to the CNS extends survival in ALS mice through modification of aberrant glial cell activity. *Molecular Therapy*, 16(6), 1056–1064. <https://doi.org/10.1038/mt.2008.60>
- Dodge, J. C., Treleaven, C. M., Fidler, J. A., Hester, M., Haidet, A., Handy, C., Rao, M., Eagle, A., Matthews, J. C., Taksir, T. V., Cheng, S. H., Shihabuddin, L. S., & Kaspar, B. K. (2010). AAV4-mediated expression of IGF-1 and VEGF within cellular components of the ventricular system improves survival outcome in familial ALS mice. *Molecular Therapy*, 18(12), 2075–2084. <https://doi.org/10.1038/mt.2010.206>
- Doi, S., Zou, Y., Togao, O., Pastor, J. V., John, G. B., Wang, L., Shiizaki, K., Gotschall, R., Schiavi, S., Yorioka, N., Takahashi, M., Boothman, D. A., & Kuro-o, M. (2011). Klotho Inhibits Transforming Growth Factor- β 1 (TGF- β 1) Signaling and Suppresses Renal Fibrosis and Cancer Metastasis in Mice. *Journal of Biological Chemistry*, 286(10), 8655–8665. <https://doi.org/10.1074/jbc.M110.174037>
- Driscoll, I., Ma, Y., Gallagher, C. L., Johnson, S. C., Asthana, S., Hermann, B. P., Sager, M. A., Blennow, K., Zetterberg, H., Carlsson, C. M., Engelman, C. D., Dubal, D. B., & Okonkwo, O. C. (2021). Age-Related Tau Burden and Cognitive Deficits Are Attenuated in KLOTHO KL-VS Heterozygotes1. *Journal of Alzheimer's Disease*, 79(3), 1297–1305. <https://doi.org/10.3233/JAD-200944>
- Duan, D. (2023). Lethal immunotoxicity in high-dose systemic AAV therapy. *Molecular Therapy*, 31(11), 3123–3126. <https://doi.org/10.1016/j.ymthe.2023.10.015>
- Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K. J., & Engelhardt, J. F. (1998). Circular Intermediates of Recombinant Adeno-Associated Virus Have Defined Structural Characteristics Responsible for Long-Term Episomal Persistence in Muscle Tissue. *Journal of Virology*, 72(11), 8568–8577. <https://doi.org/10.1128/JVI.72.11.8568-8577.1998>
- Duan, D., Yue, Y., Yan, Z., & Engelhardt, J. F. (2000). A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nature Medicine*, 6(5), 595–598. <https://doi.org/10.1038/75080>
- Duan, W., Guo, M., Yi, L., Liu, Y., Li, Z., Ma, Y., Zhang, G., Liu, Y., Bu, H., Song, X., & Li, C. (2020). The deletion of mutant SOD1 via CRISPR/Cas9/sgRNA prolongs survival in an amyotrophic lateral sclerosis mouse model. *Gene Therapy*, 27(3–4), 157–169. <https://doi.org/10.1038/s41434-019-0116-1>
- Dubal, D. B., Zhu, L., Sanchez, P. E., Worden, K., Broestl, L., Johnson, E., Ho, K., Yu, G.-Q., Kim, D., Betourne, A., Kuro-o, M., Masliah, E., Abraham, C. R., & Mucke, L. (2015). Life Extension Factor Klotho Prevents Mortality and Enhances Cognition in hAPP Transgenic Mice. *The Journal of Neuroscience*, 35(6), 2358–2371. <https://doi.org/10.1523/JNEUROSCI.5791-12.2015>
- Duce, J. A., Podvin, S., Hollander, W., Kipling, D., Rosene, D. L., & Abraham, C. R. (2008). Gene profile analysis implicates Klotho as an important contributor to aging changes in brain white matter of the rhesus monkey. *Glia*, 56(1), 106–117. <https://doi.org/10.1002/glia.20593>
- Dunbar, C. E., High, K. A., Joung, J. K., Kohn, D. B., Ozawa, K., & Sadelain, M. (2018). Gene therapy comes of age. *Science*, 359(6372). <https://doi.org/10.1126/science.aan4672>
- EDARAVONE (MCI-186) ALS 16 STUDY GROUP. (2017). A post-hoc subgroup analysis of outcomes in the first phase III clinical study of edaravone (MCI-186) in amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, 18(sup1), 11–19. <https://doi.org/10.1080/21678421.2017.1363780>
- Efron, B., & Tibshirani, R. (2007). On testing the significance of sets of genes. *The Annals of Applied Statistics*, 1(1). <https://doi.org/10.1214/07-AOAS101>
- Eisen, A., Kim, S., & Pant, B. (1992). Amyotrophic lateral sclerosis (ALS): A phylogenetic disease of the corticomotoneuron? *Muscle & Nerve*, 15(2), 219–224. <https://doi.org/10.1002/mus.880150215>

- Eisen, A., Shytbel, W., Murphy, K., & Hoirsch, M. (1990). Cortical magnetic stimulation in amyotrophic lateral sclerosis. *Muscle & Nerve*, 13(2), 146–151. <https://doi.org/10.1002/mus.880130211>
- Elden, A. C., Kim, H.-J., Hart, M. P., Chen-Plotkin, A. S., Johnson, B. S., Fang, X., Armakola, M., Geser, F., Greene, R., Lu, M. M., Padmanabhan, A., Clay-Falcone, D., McCluskey, L., Elman, L., Juhr, D., Gruber, P. J., Rüb, U., Auburger, G., Trojanowski, J. Q., ... Gitler, A. D. (2010). Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. *Nature*, 466(7310), 1069–1075. <https://doi.org/10.1038/nature09320>
- Eleftheriadou, I., Manolaras, I., Irvine, E. E., Dieringer, M., Trabalza, A., & Mazarakis, N. D. (2016). α CAR IGF-1 vector targeting of motor neurons ameliorates disease progression in ALS mice. *Annals of Clinical and Translational Neurology*, 3(10), 752–768. <https://doi.org/10.1002/acn3.335>
- Elmore, Z. C., Patrick Havlik, L., Oh, D. K., Anderson, L., Daaboul, G., Devlin, G. W., Vincent, H. A., & Asokan, A. (2021). The membrane associated accessory protein is an adeno-associated viral egress factor. *Nature Communications*, 12(1), 6239. <https://doi.org/10.1038/s41467-021-26485-4>
- Emde, A., Eitan, C., Liou, L.-L., Libby, R. T., Rivkin, N., Magen, I., Reichenstein, I., Oppenheim, H., Eilam, R., Silvestroni, A., Alajajian, B., Ben-Dov, I. Z., Aebischer, J., Savidor, A., Levin, Y., Sons, R., Hammond, S. M., Ravits, J. M., Möller, T., & Hornstein, E. (2015). Dysregulated miRNA biogenesis downstream of cellular stress and ALS-causing mutations: a new mechanism for ALS. *The EMBO Journal*, 34(21), 2633–2651. <https://doi.org/10.15252/embj.201490493>
- Endo, K., Ishigaki, S., Masamizu, Y., Fujioka, Y., Watakabe, A., Yamamori, T., Hatanaka, N., Nambu, A., Okado, H., Katsuno, M., Watanabe, H., Matsuzaki, M., & Sobue, G. (2018). Silencing of FUS in the common marmoset (*Callithrix jacchus*) brain via stereotaxic injection of an adeno-associated virus encoding shRNA. *Neuroscience Research*, 130, 56–64. <https://doi.org/10.1016/j.neures.2017.08.006>
- Ertl, H. C. J. (2022). Immunogenicity and toxicity of AAV gene therapy. *Frontiers in Immunology*, 13. <https://doi.org/10.3389/fimmu.2022.975803>
- Fadeel, B., & Grzybowska, E. (2009). HAX-1: A multifunctional protein with emerging roles in human disease. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1790(10), 1139–1148. <https://doi.org/10.1016/j.bbagen.2009.06.004>
- Fang, T., Jozsa, F., & Al-Chalabi, A. (2017). Nonmotor Symptoms in Amyotrophic Lateral Sclerosis: A Systematic Review. In *International Review of Neurobiology* (Vol. 134, pp. 1409–1441). Academic Press Inc. <https://doi.org/10.1016/bs.irn.2017.04.009>
- Feldman, E. L., Goutman, S. A., Petri, S., Mazzini, L., Savelieff, M. G., Shaw, P. J., & Sobue, G. (2022). Amyotrophic lateral sclerosis. In *The Lancet* (Vol. 400, Issue 10360, pp. 1363–1380). Elsevier B.V. [https://doi.org/10.1016/S0140-6736\(22\)01272-7](https://doi.org/10.1016/S0140-6736(22)01272-7)
- Ferrante, R. J., Browne, S. E., Shinobu, L. A., Bowling, A. C., Baik, M. J., MacGarvey, U., Kowall, N. W., Brown, R. H., & Beal, M. F. (2002). Evidence of Increased Oxidative Damage in Both Sporadic and Familial Amyotrophic Lateral Sclerosis. *Journal of Neurochemistry*, 69(5), 2064–2074. <https://doi.org/10.1046/j.1471-4159.1997.69052064.x>
- Fil, D., DeLoach, A., Yadav, S., Alkam, D., MacNicol, M., Singh, A., Compadre, C. M., Goellner, J. J., O'Brien, C. A., Fahmi, T., Basnakian, A. G., Calingasan, N. Y., Klessner, J. L., Beal, F. M., Peters, O. M., Metterville, J., Brown, R. H., Ling, K. K. Y., Rigo, F., ... Kiaei, M. (2017). Mutant Profilin1 transgenic mice recapitulate cardinal features of motor neuron disease. *Human Molecular Genetics*, 26(4), 686–701. <https://doi.org/10.1093/hmg/ddw429>
- Fiore, A. P. Z. P., Maity, S., An, D., Rendleman, J., Iannitelli, D., Choi, H., Mazzoni, E., & Vogel, C. (2023). *New molecular signatures defining the differential proteostasis response in ALS-resistant and -sensitive motor neurons*. <https://doi.org/10.1101/2022.04.10.487765>
- Fischer, L. R., Culver, D. G., Tennant, P., Davis, A. A., Wang, M., Castellano-Sanchez, A., Khan, J., Polak, M. A., & Glass, J. D. (2004a). Amyotrophic lateral sclerosis is a distal axonopathy: Evidence in mice and man. *Experimental Neurology*, 185(2), 232–240. <https://doi.org/10.1016/j.expneurol.2003.10.004>
- Fogarty, M. (2019). Amyotrophic lateral sclerosis as a synaptopathy. *Neural Regeneration Research*, 14(2), 189. <https://doi.org/10.4103/1673-5374.244782>
- Fogarty, M. J., Noakes, P. G., & Bellingham, M. C. (2015). Motor Cortex Layer V Pyramidal Neurons Exhibit Dendritic Regression, Spine Loss, and Increased Synaptic Excitation in the Presymptomatic hSOD1^{G93A} Mouse Model of Amyotrophic Lateral Sclerosis. *The Journal of Neuroscience*, 35(2), 643–647. <https://doi.org/10.1523/JNEUROSCI.3483-14.2015>

- Forster, R. E., Jurutka, P. W., Hsieh, J.-C., Haussler, C. A., Lowmiller, C. L., Kaneko, I., Haussler, M. R., & Kerr Whitfield, G. (2011). Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. *Biochemical and Biophysical Research Communications*, 414(3), 557–562. <https://doi.org/10.1016/j.bbrc.2011.09.117>
- Foust, K. D., Salazar, D. L., Likhite, S., Ferraiuolo, L., Ditsworth, D., Ilieva, H., Meyer, K., Schmelzer, L., Braun, L., Cleveland, D. W., & Kaspar, B. K. (2013a). Therapeutic AAV9-mediated suppression of mutant SOD1 slows disease progression and extends survival in models of inherited ALS. *Molecular Therapy*, 21(12), 2148–2159. <https://doi.org/10.1038/mt.2013.211>
- Franco-Iborra, S., Cuadros, T., Parent, A., Romero-Gimenez, J., Vila, M., & Perier, C. (2018). Defective mitochondrial protein import contributes to complex I-induced mitochondrial dysfunction and neurodegeneration in Parkinson's disease. *Cell Death & Disease*, 9(11), 1122. <https://doi.org/10.1038/s41419-018-1154-0>
- Franz, C. K., Federici, T., Yang, J., Backus, C., Oh, S. S., Teng, Q., Carlton, E., Bishop, K. M., Gasmi, M., Bartus, R. T., Feldman, E. L., & Boulis, N. M. (2009). Intraspinal cord delivery of IGF-I mediated by adeno-associated virus 2 is neuroprotective in a rat model of familial ALS. *Neurobiology of Disease*, 33(3), 473–481. <https://doi.org/10.1016/j.nbd.2008.12.003>
- Freibaum, B. D., Lu, Y., Lopez-Gonzalez, R., Kim, N. C., Almeida, S., Lee, K.-H., Badders, N., Valentine, M., Miller, B. L., Wong, P. C., Petrucelli, L., Kim, H. J., Gao, F.-B., & Taylor, J. P. (2015). GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. *Nature*, 525(7567), 129–133. <https://doi.org/10.1038/nature14974>
- Frey, D., Schneider, C., Xu, L., Borg, J., Spooren, W., & Caroni, P. (2000). Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *Journal of Neuroscience*, 20(7), 2534–2542. <https://doi.org/10.1523/jneurosci.20-07-02534.2000>
- Friedmann, T., & Roblin, R. (1972). Gene Therapy for Human Genetic Disease? *Science*, 175(4025), 949–955. <https://doi.org/10.1126/science.175.4025.949>
- Fuchs, A., Kutterer, S., Mühling, T., Duda, J., Schütz, B., Liss, B., Keller, B. U., & Roeper, J. (2013). Selective mitochondrial Ca²⁺ uptake deficit in disease endstage vulnerable motoneurons of the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Journal of Physiology*, 591(10), 2723–2745. <https://doi.org/10.1113/jphysiol.2012.247981>
- Fuchs, A., Ringer, C., Bilkei-Gorzo, A., Weihe, E., Roeper, J., & Schütz, B. (2010). Downregulation of the Potassium Chloride Cotransporter KCC2 in Vulnerable Motoneurons in the SOD1-G93A Mouse Model of Amyotrophic Lateral Sclerosis. *Journal of Neuropathology & Experimental Neurology*, 69(10), 1057–1070. <https://doi.org/10.1097/NEN.0b013e3181f4dcef>
- Gaj, T., Ojala, D. S., Ekman, F. K., Byrne, L. C., Limsirichai, P., & Schaffer, D. V. (2017). In vivo genome editing improves motor function and extends survival in a mouse model of ALS. *Science Advances*, 3(12). <https://doi.org/10.1126/sciadv.aar3952>
- Gal, J., Ström, A.-L., Kilty, R., Zhang, F., & Zhu, H. (2007). p62 Accumulates and Enhances Aggregate Formation in Model Systems of Familial Amyotrophic Lateral Sclerosis. *Journal of Biological Chemistry*, 282(15), 11068–11077. <https://doi.org/10.1074/jbc.M608787200>
- Galganski, L., Urbanek, M. O., & Krzyzosiak, W. J. (2017). Nuclear speckles: molecular organization, biological function and role in disease. *Nucleic Acids Research*, 45(18), 10350–10368. <https://doi.org/10.1093/nar/gkx759>
- Gallart-Palau, X., Tarabal, O., Casanovas, A., Sábado, J., Correa, F. J., Hereu, M., Piedrafita, L., Calderó, J., & Esquerda, J. E. (2014). Neuregulin-1 is concentrated in the postsynaptic subsurface cistern of C-bouton inputs to α -motoneurons and altered during motoneuron diseases. *The FASEB Journal*, 28(8), 3618–3632. <https://doi.org/10.1096/fj.13-248583>
- Gao, H., Freeling, J., Wu, P., Liang, A. P., Wang, X., & Li, Y. (2019). UCHL1 regulates muscle fibers and mTORC1 activity in skeletal muscle. *Life Sciences*, 233, 116699. <https://doi.org/10.1016/j.lfs.2019.116699>
- Gao, V., Briano, J. A., Komer, L. E., & Burré, J. (2023). Functional and Pathological Effects of α -Synuclein on Synaptic SNARE Complexes. *Journal of Molecular Biology*, 435(1), 167714. <https://doi.org/10.1016/j.jmb.2022.167714>
- García-Álías, G., Verdú, E., Forés, J., López-Vales, R., & Navarro, X. (2003). Functional and Electrophysiological Characterization of Photochemical Graded Spinal Cord Injury in the Rat. *Journal of Neurotrauma*, 20(5), 501–510. <https://doi.org/10.1089/089771503765355568>

- Ghosh, A., Yue, Y., Lai, Y., & Duan, D. (2008). A Hybrid Vector System Expands Adeno-associated Viral Vector Packaging Capacity in a Transgene-independent Manner. *Molecular Therapy*, 16(1), 124–130. <https://doi.org/10.1038/sj.mt.6300322>
- Giannini, M., Bayona-Feliu, A., Sproviero, D., Barroso, S. I., Cereda, C., & Aguilera, A. (2020). TDP-43 mutations link Amyotrophic Lateral Sclerosis with R-loop homeostasis and R loop-mediated DNA damage. *PLOS Genetics*, 16(12), e1009260. <https://doi.org/10.1371/journal.pgen.1009260>
- Gibson, G. (2022). Perspectives on rigor and reproducibility in single cell genomics. *PLOS Genetics*, 18(5), e1010210. <https://doi.org/10.1371/journal.pgen.1010210>
- Gomes, C., Escrevente, C., & Costa, J. (2010). Mutant superoxide dismutase 1 overexpression in NSC-34 cells: Effect of trehalose on aggregation, TDP-43 localization and levels of co-expressed glycoproteins. *Neuroscience Letters*, 475(3), 145–149. <https://doi.org/10.1016/j.neulet.2010.03.065>
- Gong, Y. H., Parsadanian, A. S., Andreeva, A., Snider, W. D., & Elliott, J. L. (2000). Restricted Expression of G86R Cu/Zn Superoxide Dismutase in Astrocytes Results in Astrocytosis But Does Not Cause Motoneuron Degeneration. *The Journal of Neuroscience*, 20(2), 660–665. <https://doi.org/10.1523/JNEUROSCI.20-02-00660.2000>
- Gorlewicz, A. (2009). CD44 is expressed in non-myelinating Schwann cells of the adult rat, and may play a role in neurodegeneration-induced glial plasticity at the neuromuscular junction. *Neurobiology of Disease*, 34(2), 245.
- Gould, T. W., Buss, R. R., Vinsant, S., Prevette, D., Sun, W., Knudson, C. M., Milligan, C. E., & Oppenheim, R. W. (2006). Complete Dissociation of Motor Neuron Death from Motor Dysfunction by Bax Deletion in a Mouse Model of ALS. *The Journal of Neuroscience*, 26(34), 8774–8786. <https://doi.org/10.1523/JNEUROSCI.2315-06.2006>
- Goutman, S. A., Hardiman, O., Al-Chalabi, A., Chió, A., Savelieff, M. G., Kiernan, M. C., & Feldman, E. L. (2022). Recent advances in the diagnosis and prognosis of amyotrophic lateral sclerosis. In *The Lancet Neurology* (Vol. 21, Issue 5, pp. 480–493). Elsevier Ltd. [https://doi.org/10.1016/S1474-4422\(21\)00465-8](https://doi.org/10.1016/S1474-4422(21)00465-8)
- Gray, S. J., Matagne, V., Bachaboina, L., Yadav, S., Ojeda, S. R., & Samulski, R. J. (2011). Preclinical Differences of Intravascular AAV9 Delivery to Neurons and Glia: A Comparative Study of Adult Mice and Nonhuman Primates. *Molecular Therapy*, 19(6), 1058–1069. <https://doi.org/10.1038/mt.2011.72>
- Greenway, M. J., Andersen, P. M., Russ, C., Ennis, S., Cashman, S., Donaghy, C., Patterson, V., Swingle, R., Kieran, D., Prehn, J., Morrison, K. E., Green, A., Acharya, K. R., Brown, R. H., & Hardiman, O. (2006). ANG mutations segregate with familial and “sporadic” amyotrophic lateral sclerosis. *Nature Genetics*, 38(4), 411–413. <https://doi.org/10.1038/ng1742>
- Greig, J. A., Martins, K. M., Breton, C., Lamontagne, R. J., Zhu, Y., He, Z., White, J., Zhu, J.-X., Chichester, J. A., Zheng, Q., Zhang, Z., Bell, P., Wang, L., & Wilson, J. M. (2023). Integrated vector genomes may contribute to long-term expression in primate liver after AAV administration. *Nature Biotechnology*. <https://doi.org/10.1038/s41587-023-01974-7>
- Grieger, J. C., Soltys, S. M., & Samulski, R. J. (2016). Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. *Molecular Therapy*, 24(2), 287–297. <https://doi.org/10.1038/mt.2015.187>
- Grøntvedt, G. R., Sando, S. B., Lauridsen, C., Bråthen, G., White, L. R., Salvesen, Ø., Aarsland, D., Hessen, E., Fladby, T., Waterloo, K., & Scheffler, K. (2022). Association of Klotho Protein Levels and KL-VS Heterozygosity With Alzheimer Disease and Amyloid and Tau Burden. *JAMA Network Open*, 5(11), e2243232. <https://doi.org/10.1001/jamanetworkopen.2022.43232>
- Guillot, S., Azzouz, M., Déglon, N., Zurn, A., & Aebischer, P. (2004). Local GDNF expression mediated by lentiviral vector protects facial nerve motoneurons but not spinal motoneurons in SOD1G93A transgenic mice. *Neurobiology of Disease*, 16(1), 139–149. <https://doi.org/10.1016/j.nbd.2004.01.017>
- Guo, Y., Zhang, Y., Wen, D., Duan, W., An, T., Shi, P., Wang, J., Li, Z., Chen, X., & Li, C. (2013). The modest impact of transcription factor Nrf2 on the course of disease in an ALS animal model. *Laboratory Investigation*, 93(7), 825–833. <https://doi.org/10.1038/labinvest.2013.73>
- Guzmán-Lenis, M.-S., Navarro, X., & Casas, C. (2009). Drug screening of neuroprotective agents on an organotypic-based model of spinal cord excitotoxic damage. *Restorative Neurology and Neuroscience*, 27(4), 335–349. <https://doi.org/10.3233/RNN-2009-0482>

- Haase, G., Kennel, P., Pettmann, B., Vigne, E., Akli, S., Revah, F., Schmalbruch, H., & Kahn, A. (1997). Gene therapy of murine motor neuron disease using adenoviral vectors for neurotrophic factors. *Nature Medicine*, 3(4), 429–436. <https://doi.org/10.1038/nm0497-429>
- Haase, G., Pettmann, B., Vigne, E., Castelnau-Ptakhine, L., Schmalbruch, H., & Kahn, A. (1998). Adenovirus-mediated transfer of the neurotrophin-3 gene into skeletal muscle of pmn mice: Therapeutic effects and mechanisms of action. *Journal of the Neurological Sciences*, 160, S97–S105. [https://doi.org/10.1016/S0022-510X\(98\)00207-X](https://doi.org/10.1016/S0022-510X(98)00207-X)
- Hadzipasic, M., Tahvildari, B., Nagy, M., Bian, M., Horwich, A. L., & McCormick, D. A. (2014). Selective degeneration of a physiological subtype of spinal motor neuron in mice with SOD1-linked ALS. *Proceedings of the National Academy of Sciences*, 111(47), 16883–16888. <https://doi.org/10.1073/pnas.1419497111>
- Haeusler, A. R., Donnelly, C. J., Periz, G., Simko, E. A. J., Shaw, P. G., Kim, M.-S., Maragakis, N. J., Troncoso, J. C., Pandey, A., Sattler, R., Rothstein, J. D., & Wang, J. (2014). C9orf72 nucleotide repeat structures initiate molecular cascades of disease. *Nature*, 507(7491), 195–200. <https://doi.org/10.1038/nature13124>
- Haidet-Phillips, A. M., Hester, M. E., Miranda, C. J., Meyer, K., Braun, L., Frakes, A., Song, S., Likhite, S., Murtha, M. J., Foust, K. D., Rao, M., Eagle, A., Kammesheid, A., Christensen, A., Mendell, J. R., Burghes, A. H. M., & Kaspar, B. K. (2011). Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nature Biotechnology*, 29(9), 824–828. <https://doi.org/10.1038/nbt.1957>
- Halter, B., Gonzalez de Aguilar, J.-L., Rene, F., Petri, S., Fricker, B., Echaniz-Laguna, A., Dupuis, L., Larmet, Y., & Loeffler, J.-P. (2010). Oxidative stress in skeletal muscle stimulates early expression of Rad in a mouse model of amyotrophic lateral sclerosis. *Free Radical Biology and Medicine*, 48(7), 915–923. <https://doi.org/10.1016/j.freeradbiomed.2010.01.014>
- Harborne, S. P. D., King, M. S., Crichton, P. G., & Kunji, E. R. S. (2017). Calcium regulation of the human mitochondrial ATP-Mg/Pi carrier SLC25A24 uses a locking pin mechanism. *Scientific Reports*, 7(1), 45383. <https://doi.org/10.1038/srep45383>
- Hassan, M. T., & Lytton, J. (2020). Potassium-dependent sodium-calcium exchanger (NCKX) isoforms and neuronal function. *Cell Calcium*, 86, 102135. <https://doi.org/10.1016/j.ceca.2019.102135>
- Hatzipetros, T., Kidd, J. D., Moreno, A. J., Thompson, K., Gill, A., & Vieira, F. G. (2015). A Quick Phenotypic Neurological Scoring System for Evaluating Disease Progression in the SOD1-G93A Mouse Model of ALS. *Journal of Visualized Experiments*, 104. <https://doi.org/10.3791/53257>
- Hawrot, J., Imhof, S., & Wainger, B. J. (2020). Modeling cell-autonomous motor neuron phenotypes in ALS using iPSCs. *Neurobiology of Disease*, 134, 104680. <https://doi.org/10.1016/j.nbd.2019.104680>
- Hedlund, E., Karlsson, M., Osborn, T., Ludwig, W., & Isacson, O. (2010). Global gene expression profiling of somatic motor neuron populations with different vulnerability identify molecules and pathways of degeneration and protection. *Brain*, 133(8), 2313–2330. <https://doi.org/10.1093/brain/awq167>
- Hegedus, J., Putman, C. T., & Gordon, T. (2007). Time course of preferential motor unit loss in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Neurobiology of Disease*, 28(2), 154–164. <https://doi.org/10.1016/j.nbd.2007.07.003>
- Hendricks, A. L., Wachnowsky, C., Fries, B., Fidai, I., & Cowan, J. A. (2021). Characterization and Reconstitution of Human Lipoyl Synthase (LIAS) Supports ISCA2 and ISCU as Primary Cluster Donors and an Ordered Mechanism of Cluster Assembly. *International Journal of Molecular Sciences*, 22(4), 1598. <https://doi.org/10.3390/ijms22041598>
- Henkel, J. S., Beers, D. R., Zhao, W., & Appel, S. H. (2009). Microglia in ALS: The good, the bad, and the resting. *Journal of Neuroimmune Pharmacology*, 4(4), 389–398. <https://doi.org/10.1007/s11481-009-9171-5>
- Henriques, A., Pitzer, C., Dittgen, T., Klugmann, M., Dupuis, L., & Schneider, A. (2011). CNS-targeted viral delivery of G-CSF in an animal model for ALS: Improved efficacy and preservation of the neuromuscular unit. *Molecular Therapy*, 19(2), 284–292. <https://doi.org/10.1038/mt.2010.271>
- Hensley, K., Mhatre, M., Mou, S., Pye, Q. N., Stewart, C., West, M., & Williamson, K. S. (2006). On the relation of oxidative stress to neuroinflammation: Lessons learned from the G93A-SOD1 mouse model of amyotrophic lateral sclerosis. *Antioxidants and Redox Signaling*, 8(11–12), 2075–2087. <https://doi.org/10.1089/ars.2006.8.2075>
- Herrando-Grabulosa, M., Mulet, R., Pujol, A., Mas, J. M., Navarro, X., Aloy, P., Coma, M., & Casas, C. (2016). Novel Neuroprotective Multicomponent Therapy for Amyotrophic Lateral Sclerosis Designed by Networked Systems. *PLOS ONE*, 11(1), e0147626. <https://doi.org/10.1371/journal.pone.0147626>

- Hetz, C. (2012). The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews Molecular Cell Biology*, 13(2), 89–102. <https://doi.org/10.1038/nrm3270>
- Hewitt, C., Kirby, J., Highley, J. R., Hartley, J. A., Hibberd, R., Hollinger, H. C., Williams, T. L., Ince, P. G., McDermott, C. J., & Shaw, P. J. (2010). Novel FUS/TLS Mutations and Pathology in Familial and Sporadic Amyotrophic Lateral Sclerosis. *Archives of Neurology*, 67(4). <https://doi.org/10.1001/archneurol.2010.52>
- Hirano, A., Donnenfeld, H., Shoichi, S., & Nakano, I. (1984). Fine Structural Observations of Neurofilamentous Changes in Amyotrophic Lateral Sclerosis. *Journal of Neuropathology and Experimental Neurology*, 43(5), 461–470.
- Hitoshi, N., Ken-ichi, Y., & Jun-ichi, M. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, 108(2), 193–199. [https://doi.org/10.1016/0378-1119\(91\)90434-D](https://doi.org/10.1016/0378-1119(91)90434-D)
- Homs, J., Pagès, G., Ariza, L., Casas, C., Chillón, M., Navarro, X., & Bosch, A. (2014). Intrathecal administration of IGF-I by AAVrh10 improves sensory and motor deficits in a mouse model of diabetic neuropathy. *Molecular Therapy - Methods and Clinical Development*, 1, 7. <https://doi.org/10.1038/mtm.2013.7>
- Howland, D. S., Liu, J., She, Y., Goad, B., Maragakis, N. J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., DeGennaro, L. J., Cleveland, D. W., & Rothstein, J. D. (2002). Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proceedings of the National Academy of Sciences of the United States of America*, 99(3), 1604–1609. <https://doi.org/10.1073/pnas.032539299>
- Hu, J., Liu, J., Li, L., Gagnon, K. T., & Corey, D. R. (2015). Engineering Duplex RNAs for Challenging Targets: Recognition of GGGGCC/CCCCGG Repeats at the ALS/FTD C9orf72 Locus. *Chemistry & Biology*, 22(11), 1505–1511. <https://doi.org/10.1016/j.chembiol.2015.09.016>
- Hu, J., Su, B., Li, X., Li, Y., & Zhao, J. (2021). Klotho overexpression suppresses apoptosis by regulating the Hsp70/Akt/Bad pathway in H9c2(2-1) cells. *Experimental and Therapeutic Medicine*, 21(5), 486. <https://doi.org/10.3892/etm.2021.9917>
- Hu, M. C., Shi, M., Zhang, J., Addo, T., Cho, H. J., Barker, S. L., Ravikumar, P., Gillings, N., Bian, A., Sidhu, S. S., Kuro-o, M., & Moe, O. W. (2016). Renal Production, Uptake, and Handling of Circulating αKlotho. *Journal of the American Society of Nephrology*, 27(1), 79–90. <https://doi.org/10.1681/ASN.2014101030>
- Huang, S.-L., Wu, L.-S., Lee, M., Chang, C.-W., Cheng, W.-C., Fang, Y.-S., Chen, Y.-R., Cheng, P.-L., & Shen, C.-K. J. (2020). A robust TDP-43 knock-in mouse model of ALS. *Acta Neuropathologica Communications*, 8(1), 3. <https://doi.org/10.1186/s40478-020-0881-5>
- Iannitti, T., Scarrott, J. M., Likhite, S., Coldicott, I. R. P., Lewis, K. E., Heath, P. R., Higginbottom, A., Myszczyńska, M. A., Milo, M., Hautbergue, G. M., Meyer, K., Kaspar, B. K., Ferraiuolo, L., Shaw, P. J., & Azzouz, M. (2018). Translating SOD1 Gene Silencing toward the Clinic: A Highly Efficacious, Off-Target-free, and Biomarker-Supported Strategy for fALS. *Molecular Therapy. Nucleic Acids*, 12, 75–88. <https://doi.org/10.1016/j.omtn.2018.04.015>
- Iijima, H., Gilmer, G., Wang, K., Bean, A. C., He, Y., Lin, H., Tang, W.-Y., Lamont, D., Tai, C., Ito, A., Jones, J. J., Evans, C., & Ambrosio, F. (2023). Age-related matrix stiffening epigenetically regulates α-Klotho expression and compromises chondrocyte integrity. *Nature Communications*, 14(1), 18. <https://doi.org/10.1038/s41467-022-35359-2>
- Ikushima, M., Rakugi, H., Ishikawa, K., Maekawa, Y., Yamamoto, K., Ohta, J., Chihara, Y., Kida, I., & Ogihara, T. (2006). Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells. *Biochemical and Biophysical Research Communications*, 339(3), 827–832. <https://doi.org/10.1016/j.bbrc.2005.11.094>
- Imura, A., Iwano, A., Tohyama, O., Tsuji, Y., Nozaki, K., Hashimoto, N., Fujimori, T., & Nabeshima, Y.-I. (2004). Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Letters*, 565(1–3), 143–147. <https://doi.org/10.1016/j.febslet.2004.03.090>
- Isakova, T., Wahl, P., Vargas, G. S., Gutiérrez, O. M., Scialla, J., Xie, H., Appleby, D., Nessel, L., Bellovich, K., Chen, J., Hamm, L., Gadegbeku, C., Horwitz, E., Townsend, R. R., Anderson, C. A. M., Lash, J. P., Hsu, C., Leonard, M. B., & Wolf, M. (2011). Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney International*, 79(12), 1370–1378. <https://doi.org/10.1038/ki.2011.47>
- Ito, S., Fujimori, T., Hayashizaki, Y., & Nabeshima, Y. (2002). Identification of a novel mouse membrane-bound family 1 glycosidase-like protein, which carries an atypical active site structure. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, 1576(3), 341–345. [https://doi.org/10.1016/S0167-4781\(02\)00281-6](https://doi.org/10.1016/S0167-4781(02)00281-6)

- Ito, S., Kinoshita, S., Shiraishi, N., Nakagawa, S., Sekine, S., Fujimori, T., & Nabeshima, Y. (2000). Molecular cloning and expression analyses of mouse β klotho, which encodes a novel Klotho family protein. *Mechanisms of Development*, 98(1–2), 115–119. [https://doi.org/10.1016/S0925-4773\(00\)00439-1](https://doi.org/10.1016/S0925-4773(00)00439-1)
- Ito, Y., Yamada, M., Tanaka, H., Aida, K., Tsuruma, K., Shimazawa, M., Hozumi, I., Inuzuka, T., Takahashi, H., & Hara, H. (2009). Involvement of CHOP, an ER-stress apoptotic mediator, in both human sporadic ALS and ALS model mice. *Neurobiology of Disease*, 36(3), 470–476. <https://doi.org/10.1016/j.nbd.2009.08.013>
- Iwama, K., Osaka, H., Ikeda, T., Mitsuhashi, S., Miyatake, S., Takata, A., Miyake, N., Ito, S., Mizuguchi, T., & Matsumoto, N. (2018). A novel SLC9A1 mutation causes cerebellar ataxia. *Journal of Human Genetics*, 63(10), 1049–1054. <https://doi.org/10.1038/s10038-018-0488-x>
- Jara, J. H., Villa, S. R., Khan, N. A., Bohn, M. C., & Özdinler, P. H. (2012). AAV2 mediated retrograde transduction of corticospinal motor neurons reveals initial and selective apical dendrite degeneration in ALS. *Neurobiology of Disease*, 47(2), 174–183. <https://doi.org/10.1016/j.nbd.2012.03.036>
- Jiang, J., Taylor, D. K., Kim, E. J., Boyd, L. F., Ahmad, J., Mage, M. G., Truong, H. V., Woodward, C. H., Sgourakis, N. G., Cresswell, P., Margulies, D. H., & Natarajan, K. (2022). Structural mechanism of tapasin-mediated MHC-I peptide loading in antigen presentation. *Nature Communications*, 13(1), 5470. <https://doi.org/10.1038/s41467-022-33153-8>
- Jiang, J., Zhu, Q., Gendron, T. F., Saberi, S., McAlonis-Downes, M., Seelman, A., Stauffer, J. E., Jafar-nejad, P., Drenner, K., Schulte, D., Chun, S., Sun, S., Ling, S.-C., Myers, B., Engelhardt, J., Katz, M., Baughn, M., Platoshyn, O., Marsala, M., ... Lagier-Tourenne, C. (2016). Gain of Toxicity from ALS/FTD-Linked Repeat Expansions in C9ORF72 Is Alleviated by Antisense Oligonucleotides Targeting GGGGCC-Containing RNAs. *Neuron*, 90(3), 535–550. <https://doi.org/10.1016/j.neuron.2016.04.006>
- Jo, M., Lee, S., Jeon, Y.-M., Kim, S., Kwon, Y., & Kim, H.-J. (2020). The role of TDP-43 propagation in neurodegenerative diseases: integrating insights from clinical and experimental studies. *Experimental & Molecular Medicine*, 52(10), 1652–1662. <https://doi.org/10.1038/s12276-020-00513-7>
- Jokic, N., Gonzalez de Aguilar, J.-L., Pradat, P.-F., Dupuis, L., Echaniz-Laguna, A., Muller, A., Dubourg, O., Seilhean, D., Hauw, J.-J., Loeffler, J.-P., & Meininger, V. (2005). Nogo expression in muscle correlates with amyotrophic lateral sclerosis severity. *Annals of Neurology*, 57(4), 553–556. <https://doi.org/10.1002/ana.20420>
- Jovičić, A., Mertens, J., Boeynaems, S., Bogaert, E., Chai, N., Yamada, S. B., Paul, J. W., Sun, S., Herdy, J. R., Bieri, G., Kramer, N. J., Gage, F. H., Van Den Bosch, L., Robberecht, W., & Gitler, A. D. (2015). Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS. *Nature Neuroscience*, 18(9), 1226–1229. <https://doi.org/10.1038/nn.4085>
- Kabuta, T., Kinugawa, A., Tsuchiya, Y., Kabuta, C., Setsuie, R., Tateno, M., Araki, T., & Wada, K. (2009). Familial amyotrophic lateral sclerosis-linked mutant SOD1 aberrantly interacts with tubulin. *Biochemical and Biophysical Research Communications*, 387(1), 121–126. <https://doi.org/10.1016/j.bbrc.2009.06.138>
- Kaneko, M., Noguchi, T., Ikegami, S., Sakurai, T., Kakita, A., Toyoshima, Y., Kambe, T., Yamada, M., Inden, M., Hara, H., Oyanagi, K., Inuzuka, T., Takahashi, H., & Hozumi, I. (2015). Zinc transporters ZnT3 and ZnT6 are downregulated in the spinal cords of patients with sporadic amyotrophic lateral sclerosis. *Journal of Neuroscience Research*, 93(2), 370–379. <https://doi.org/10.1002/jnr.23491>
- Kang, S. H., Li, Y., Fukaya, M., Lorenzini, I., Cleveland, D. W., Ostrow, L. W., Rothstein, J. D., & Bergles, D. E. (2013). Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nature Neuroscience*, 16(5), 571–579. <https://doi.org/10.1038/nn.3357>
- Kantor, B., Bailey, R. M., Wimberly, K., Kalburgi, S. N., & Gray, S. J. (2014). *Methods for Gene Transfer to the Central Nervous System* (pp. 125–197). <https://doi.org/10.1016/B978-0-12-800149-3.00003-2>
- Kaspar, B. K., Lladó, J., Sherkat, N., Rothstein, J. D., & Gage, F. H. (2003). Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science (New York, N.Y.)*, 301(5634), 839–842. <https://doi.org/10.1126/science.1086137>
- Kato, Y., Arakawa, E., Kinoshita, S., Shirai, A., Furuya, A., Yamano, K., Nakamura, K., Iida, A., Anazawa, H., Koh, N., Iwano, A., Imura, A., Fujimori, T., Kuro-o, M., Hanai, N., Takeshige, K., & Nabeshima, Y. (2000). Establishment of the Anti-Klotho Monoclonal Antibodies and Detection of Klotho Protein in Kidneys. *Biochemical and Biophysical Research Communications*, 267(2), 597–602. <https://doi.org/10.1006/bbrc.1999.2009>
- Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., & Kwak, S. (2004). Glutamate receptors: RNA editing and death of motor neurons. *Nature*, 427(6977), 801. <https://doi.org/10.1038/427801a>

- Keeler, A. M., Zieger, M., Semple, C., Pucci, L., Veinbachs, A., Brown, R. H., Mueller, C., & ElMallah, M. K. (2020). Intralingual and Intrapleural AAV Gene Therapy Prolongs Survival in a SOD1 ALS Mouse Model. *Molecular Therapy - Methods and Clinical Development*, 17(June), 246–257. <https://doi.org/10.1016/j.omtm.2019.12.007>
- Kellogg, C. M., Pham, K., Machalinski, A. H., Porter, H. L., Blankenship, H. E., Tooley, K., Stout, M. B., Rice, H. C., Sharpe, A. L., Beckstead, M. J., Chucair-Elliott, A. J., Ocañas, S. R., & Freeman, W. M. (2023). *Microglial MHC-I induction with aging and Alzheimer's is conserved in mouse models and humans*. <https://doi.org/10.1101/2023.03.07.531435>
- Khademullah, C. S., Bourbonnais, J., Chaineau, M. M., Castellanos-Montiel, M. J., Keramidis, I., Legault, A., Paquet, M.-É., Abrahao, A., Zinman, L., Robertson, J., Durcan, T. M., Woodin, M. A., Godin, A. G., & De Koninck, Y. (2023). *KCC2 as a novel biomarker and therapeutic target for motoneuron degenerative disease*. <https://doi.org/10.1101/2023.08.24.554410>
- Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., Burrell, J. R., & Zoing, M. C. (2011). Amyotrophic lateral sclerosis. *The Lancet*, 377(9769), 942–955. [https://doi.org/10.1016/S0140-6736\(10\)61156-7](https://doi.org/10.1016/S0140-6736(10)61156-7)
- Kim, H. J., Kim, N. C., Wang, Y.-D., Scarborough, E. A., Moore, J., Diaz, Z., MacLea, K. S., Freibaum, B., Li, S., Molliex, A., Kanagaraj, A. P., Carter, R., Boylan, K. B., Wojtas, A. M., Rademakers, R., Pinkus, J. L., Greenberg, S. A., Trojanowski, J. Q., Traynor, B. J., ... Taylor, J. P. (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature*, 495(7442), 467–473. <https://doi.org/10.1038/nature11922>
- Kim, H.-J., Raphael, A. R., LaDow, E. S., McGurk, L., Weber, R. A., Trojanowski, J. Q., Lee, V. M.-Y., Finkbeiner, S., Gitler, A. D., & Bonini, N. M. (2014). Therapeutic modulation of eIF2 α phosphorylation rescues TDP-43 toxicity in amyotrophic lateral sclerosis disease models. *Nature Genetics*, 46(2), 152–160. <https://doi.org/10.1038/ng.2853>
- Kim, S., Chung, A., Na, J., Lee, S., Jeong, S., Kim, E., Sun, W., Rhyu, I., & Park, H. (2019). Myelin degeneration induced by mutant superoxide dismutase 1 accumulation promotes amyotrophic lateral sclerosis. *Glia*, 67(10), 1910.
- King, G. D., Rosene, D. L., & Abraham, C. R. (2012). Promoter methylation and age-related downregulation of Klotho in rhesus monkey. *AGE*, 34(6), 1405–1419. <https://doi.org/10.1007/s11357-011-9315-4>
- Klein, S. M., Behrstock, S., McHugh, J., Hoffmann, K., Wallace, K., Suzuki, M., Aebischer, P., & Svendsen, C. N. (2005). GDNF Delivery Using Human Neural Progenitor Cells in a Rat Model of ALS. *Human Gene Therapy*, 16(4), 509–521. <https://doi.org/10.1089/hum.2005.16.509>
- Kohn, D. B., Chen, Y. Y., & Spencer, M. J. (2023). Successes and challenges in clinical gene therapy. *Gene Therapy*, 30(10–11), 738–746. <https://doi.org/10.1038/s41434-023-00390-5>
- Kok, J. R., Palminha, N. M., Dos Santos Souza, C., El-Khamisy, S. F., & Ferraiuolo, L. (2021). DNA damage as a mechanism of neurodegeneration in ALS and a contributor to astrocyte toxicity. *Cellular and Molecular Life Sciences*, 78(15), 5707–5729. <https://doi.org/10.1007/s00018-021-03872-0>
- Korobeynikov, V. A., Lyashchenko, A. K., Blanco-Redondo, B., Jafar-Nejad, P., & Shneider, N. A. (2022). Antisense oligonucleotide silencing of FUS expression as a therapeutic approach in amyotrophic lateral sclerosis. *Nature Medicine*, 28(1), 104–116. <https://doi.org/10.1038/s41591-021-01615-z>
- Kosakai, A., Ito, D., Nihei, Y., Yamashita, S., Okada, Y., Takahashi, K., & Suzuki, N. (2011). Degeneration of mesencephalic dopaminergic neurons in klotho mouse related to vitamin D exposure. *Brain Research*, 1382, 109–117. <https://doi.org/10.1016/j.brainres.2011.01.056>
- Kotin, R. M., Menninger, J. C., Ward, D. C., & Berns, K. I. (1991). Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics*, 10(3), 831–834. [https://doi.org/10.1016/0888-7543\(91\)90470-Y](https://doi.org/10.1016/0888-7543(91)90470-Y)
- Kraemer, B. C., Schuck, T., Wheeler, J. M., Robinson, L. C., Trojanowski, J. Q., Lee, V. M. Y., & Schellenberg, G. D. (2010). Loss of murine TDP-43 disrupts motor function and plays an essential role in embryogenesis. *Acta Neuropathologica*, 119(4), 409–419. <https://doi.org/10.1007/s00401-010-0659-0>
- Kraft, A., Resch, J., Johnson, D., & Johnson, J. (2007). Activation of the Nrf2–ARE pathway in muscle and spinal cord during ALS-like pathology in mice expressing mutant SOD1. *Experimental Neurology*, 207(1), 107–117. <https://doi.org/10.1016/j.expneurol.2007.05.026>
- Krakora, D., Mulcrone, P., Meyer, M., Lewis, C., Bernau, K., Gowing, G., Zimprich, C., Aebischer, P., Svendsen, C. N., & Suzuki, M. (2013). Synergistic Effects of GDNF and VEGF on Lifespan and Disease Progression in a Familial ALS Rat Model. *Molecular Therapy*, 21(8), 1602–1610. <https://doi.org/10.1038/mt.2013.108>

- Krasnianski, A., Deschauer, M., Neudecker, S., Gellerich, F. N., Müller, T., Schoser, B. G., Krasnianski, M., & Zierz, S. (2005). Mitochondrial changes in skeletal muscle in amyotrophic lateral sclerosis and other neurogenic atrophies. *Brain*, 128(8), 1870–1876. <https://doi.org/10.1093/brain/awh540>
- Kremer, E. J., & Perricaudet, M. (1995). Adenovirus and adeno-associated virus mediated gene transfer. *British Medical Bulletin*, 51(1), 31–44. <https://doi.org/10.1093/oxfordjournals.bmb.a072951>
- Krishnan, G., Zhang, Y., Gu, Y., Kankel, M. W., Gao, F. B., & Almeida, S. (2020). CRISPR deletion of the C9ORF72 promoter in ALS/FTD patient motor neurons abolishes production of dipeptide repeat proteins and rescues neurodegeneration. *Acta Neuropathologica*, 140(1), 81–84. <https://doi.org/10.1007/s00401-020-02154-6>
- Kuhle, J., Lindberg, R. L. P., Regeniter, A., Mehling, M., Steck, A. J., Kappos, L., & Czaplinski, A. (2009). Increased levels of inflammatory chemokines in amyotrophic lateral sclerosis. *European Journal of Neurology*, 16(6), 771–774. <https://doi.org/10.1111/j.1468-1331.2009.02560.x>
- Kumar, S. R., Markusic, D. M., Biswas, M., High, K. A., & Herzog, R. W. (2016). Clinical development of gene therapy: results and lessons from recent successes. *Molecular Therapy - Methods & Clinical Development*, 3, 16034. <https://doi.org/10.1038/mtm.2016.34>
- Kurashige, T., Morino, H., Murao, T., Izumi, Y., Sugiura, T., Kuraoka, K., Kawakami, H., Torii, T., & Maruyama, H. (2022). TDP-43 Accumulation Within Intramuscular Nerve Bundles of Patients With Amyotrophic Lateral Sclerosis. *JAMA Neurology*, 79(7), 693. <https://doi.org/10.1001/jamaneuro.2022.1113>
- Kurita, H., Okuda, R., Yokoo, K., Inden, M., & Hozumi, I. (2016). Protective roles of SLC30A3 against endoplasmic reticulum stress via ERK1/2 activation. *Biochemical and Biophysical Research Communications*, 479(4), 853–859. <https://doi.org/10.1016/j.bbrc.2016.09.119>
- Kurita, H., Yabe, S., Ueda, T., Inden, M., Kakita, A., & Hozumi, I. (2020). MicroRNA-5572 Is a Novel MicroRNA-Regulating SLC30A3 in Sporadic Amyotrophic Lateral Sclerosis. *International Journal of Molecular Sciences*, 21(12), 4482. <https://doi.org/10.3390/ijms21124482>
- Kuro-o, M. (2011). Klotho and the aging process. *The Korean Journal of Internal Medicine*, 26(2), 113–122. <https://doi.org/10.3904/kjim.2011.26.2.113>
- Kuro-O, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kumek, E., Iwasakik, H., Iida, A., Shiraki-Iida, T., Nishikawa#, S., Nagai, R., & Yo-Ichi Nabeshima, &. (1997). Mutation of the mouse klotho gene leads to a syndrome resembling ageing. In *NATURE* (Vol. 390).
- Kurosu, H., Yamamoto, M., Clark, J. D., Pastor, J. V., Nandi, A., Gurnani, P., McGuinness, O. P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., Shimomura, I., Takayama, Y., Herz, J., Kahn, C. R., Rosenblatt, K. P., & Kuro-o, M. (2005). Suppression of Aging in Mice by the Hormone Klotho. *Science*, 309(5742), 1829–1833. <https://doi.org/10.1126/science.1112766>
- Kwak, S., Hideyama, T., Yamashita, T., & Aizawa, H. (2010). AMPA receptor-mediated neuronal death in sporadic ALS. *Neuropathology: Official Journal of the Japanese Society of Neuropathology*, 30(2), 182–188. <https://doi.org/10.1111/j.1440-1789.2009.01090.x>
- Kwan, T., Kazamel, M., Thoenes, K., Si, Y., Jiang, N., & King, P. H. (2020). Wnt antagonist FRZB is a muscle biomarker of denervation atrophy in amyotrophic lateral sclerosis. *Scientific Reports*, 10(1), 16679. <https://doi.org/10.1038/s41598-020-73845-z>
- Kwiatkowski, T. J., Bosco, D. A., LeClerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E. J., Munsat, T., Valdmanis, P., Rouleau, G. A., Hosler, B. A., Cortelli, P., de Jong, P. J., Yoshinaga, Y., Haines, J. L., Pericak-Vance, M. A., Yan, J., ... Brown, R. H. (2009). Mutations in the *FUS/TLS* Gene on Chromosome 16 Cause Familial Amyotrophic Lateral Sclerosis. *Science*, 323(5918), 1205–1208. <https://doi.org/10.1126/science.1166066>
- Lafarga, V., Sirozh, O., Díaz-López, I., Galarreta, A., Hisaoka, M., Zarzuela, E., Boskovic, J., Jovanovic, B., Fernandez-Leiro, R., Muñoz, J., Stoecklin, G., Ventoso, I., & Fernandez-Capetillo, O. (2021). Widespread displacement of DNA- and RNA-binding factors underlies toxicity of arginine-rich cell-penetrating peptides. *The EMBO Journal*, 40(13). <https://doi.org/10.15252/embj.2019103311>
- Lagier-Tourenne, C., Baughn, M., Rigo, F., Sun, S., Liu, P., Li, H.-R., Jiang, J., Watt, A. T., Chun, S., Katz, M., Qiu, J., Sun, Y., Ling, S.-C., Zhu, Q., Polymenidou, M., Drenner, K., Artates, J. W., McAlonis-Downes, M., Markmiller, S., ... Ravits, J. (2013). Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. *Proceedings of the National Academy of Sciences*, 110(47). <https://doi.org/10.1073/pnas.1318835110>

- Lagier-Tourenne, C., Polymenidou, M., Hutt, K. R., Vu, A. Q., Baughn, M., Huelga, S. C., Clutario, K. M., Ling, S.-C., Liang, T. Y., Mazur, C., Wancewicz, E., Kim, A. S., Watt, A., Freier, S., Hicks, G. G., Donohue, J. P., Shiue, L., Bennett, C. F., Ravits, J., ... Yeo, G. W. (2012). Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. *Nature Neuroscience*, 15(11), 1488–1497. <https://doi.org/10.1038/nn.3230>
- Lai, Y., Yue, Y., Liu, M., Ghosh, A., Engelhardt, J. F., Chamberlain, J. S., & Duan, D. (2005). Efficient in vivo gene expression by trans-splicing adeno-associated viral vectors. *Nature Biotechnology*, 23(11), 1435–1439. <https://doi.org/10.1038/nbt1153>
- Lam, J. K. W., Chow, M. Y. T., Zhang, Y., & Leung, S. W. S. (2015). siRNA Versus miRNA as Therapeutics for Gene Silencing. *Molecular Therapy - Nucleic Acids*, 4, e252. <https://doi.org/10.1038/mtna.2015.23>
- Lambert-Smith, I. A., Saunders, D. N., & Yerbury, J. J. (2022). Proteostasis impairment and ALS. *Progress in Biophysics and Molecular Biology*, 174, 3–27. <https://doi.org/10.1016/j.pbiomolbio.2022.06.001>
- Landry, T., Li, P., Shookster, D., Jiang, Z., Li, H., Laing, B. T., Bunner, W., Langton, T., Tong, Q., & Huang, H. (2021). Centrally circulating α -klotho inversely correlates with human obesity and modulates arcuate cell populations in mice. *Molecular Metabolism*, 44, 101136. <https://doi.org/10.1016/j.molmet.2020.101136>
- Lasiene, J., Komine, O., Fujimori-Tonou, N., Powers, B., Endo, F., Watanabe, S., Shijie, J., Ravits, J., Horner, P., Misawa, H., & Yamanaka, K. (2016). Neuregulin 1 confers neuroprotection in SOD1-linked amyotrophic lateral sclerosis mice via restoration of C-boutons of spinal motor neurons. *Acta Neuropathologica Communications*, 4, 15. <https://doi.org/10.1186/s40478-016-0286-7>
- Laure, L., Suel, L., Roudaut, C., Bourg, N., Ouali, A., Bartoli, M., Richard, I., & Danièle, N. (2009). Cardiac ankyrin repeat protein is a marker of skeletal muscle pathological remodelling. *The FEBS Journal*, 276(3), 669–684. <https://doi.org/10.1111/j.1742-4658.2008.06814.x>
- Lee, D.-Y., Jeon, G. S., & Sung, J.-J. (2020). ALS-Linked Mutant SOD1 Associates with TIA-1 and Alters Stress Granule Dynamics. *Neurochemical Research*, 45(12), 2884–2893. <https://doi.org/10.1007/s11064-020-03137-5>
- Lee, J., Tsogbadrakh, B., Yang, S., Ryu, H., Kang, E., Kang, M., Kang, H. G., Ahn, C., & Oh, K.-H. (2021). Klotho ameliorates diabetic nephropathy via LKB1-AMPK-PGC1 α -mediated renal mitochondrial protection. *Biochemical and Biophysical Research Communications*, 534, 1040–1046. <https://doi.org/10.1016/j.bbrc.2020.10.040>
- Lee, S. H., Kim, S., Lee, N., Lee, J., Yu, S. S., Kim, J. H., & Kim, S. (2019). Intrathecal delivery of recombinant AAV1 encoding hepatocyte growth factor improves motor functions and protects neuromuscular system in the nerve crush and SOD1-G93A transgenic mouse models. *Acta Neuropathologica Communications*, 7(1), 96. <https://doi.org/10.1186/s40478-019-0737-z>
- Lee, S. H., Lee, N., Kim, S., Lee, J., Choi, W., Yu, S. S., Kim, J. H., & Kim, S. (2019). Intramuscular delivery of HGF-expressing recombinant AAV improves muscle integrity and alleviates neurological symptoms in the nerve crush and SOD1-G93A transgenic mouse models. *Biochemical and Biophysical Research Communications*, 517(3), 452–457. <https://doi.org/10.1016/j.bbrc.2019.07.105>
- Lee, Y., Morrison, B. M., Li, Y., Lengacher, S., Farah, M. H., Hoffman, P. N., Liu, Y., Tsingalia, A., Jin, L., Zhang, P.-W., Pellerin, L., Magistretti, P. J., & Rothstein, J. D. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature*, 487(7408), 443–448. <https://doi.org/10.1038/nature11314>
- Lee, Y.-B., Chen, H.-J., Peres, J. N., Gomez-Deza, J., Attig, J., Štalekar, M., Troakes, C., Nishimura, A. L., Scotter, E. L., Vance, C., Adachi, Y., Sardone, V., Miller, J. W., Smith, B. N., Gallo, J.-M., Ule, J., Hirth, F., Rogelj, B., Houart, C., & Shaw, C. E. (2013). Hexanucleotide Repeats in ALS/FTD Form Length-Dependent RNA Foci, Sequester RNA Binding Proteins, and Are Neurotoxic. *Cell Reports*, 5(5), 1178–1186. <https://doi.org/10.1016/j.celrep.2013.10.049>
- Lemon, R. N., & Griffiths, J. (2005). Comparing the function of the corticospinal system in different species: Organizational differences for motor specialization? *Muscle & Nerve*, 32(3), 261–279. <https://doi.org/10.1002/mus.20333>
- Leon, J., Moreno, A. J., Garay, B. I., Chalkley, R. J., Burlingame, A. L., Wang, D., & Dubal, D. B. (2017). Peripheral Elevation of a Klotho Fragment Enhances Brain Function and Resilience in Young, Aging, and α -Synuclein Transgenic Mice. *Cell Reports*, 20(6), 1360–1371. <https://doi.org/10.1016/j.celrep.2017.07.024>
- Lepore, A. C., Haenggeli, C., Gasmi, M., Bishop, K. M., Bartus, R. T., Maragakis, N. J., & Rothstein, J. D. (2007). Intraparenchymal spinal cord delivery of adeno-associated virus IGF-1 is protective in the SOD1G93A model of ALS. *Brain Research*, 1185(1), 256–265. <https://doi.org/10.1016/j.brainres.2007.09.034>

- Lepore, A. C., Rauck, B., Dejea, C., Pardo, A. C., Rao, M. S., Rothstein, J. D., & Maragakis, N. J. (2008). Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nature Neuroscience*, 11(11), 1294–1301. <https://doi.org/10.1038/nn.2210>
- Levine, T. P., Daniels, R. D., Gatta, A. T., Wong, L. H., & Hayes, M. J. (2013). The product of C9orf72, a gene strongly implicated in neurodegeneration, is structurally related to DENN Rab-GEFs. *Bioinformatics (Oxford, England)*, 29(4), 499–503. <https://doi.org/10.1093/bioinformatics/bts725>
- Leyton-Jaimes, M. F., Kahn, J., & Israelson, A. (2019a). AAV2/9-mediated overexpression of MIF inhibits SOD1 misfolding, delays disease onset, and extends survival in mouse models of ALS. *Proceedings of the National Academy of Sciences of the United States of America*, 116(29), 14755–14760. <https://doi.org/10.1073/pnas.1904665116>
- Li, D., Jing, D., Liu, Z., Chen, Y., Huang, F., & Behnisch, T. (2019). Enhanced Expression of Secreted α -Klotho in the Hippocampus Alters Nesting Behavior and Memory Formation in Mice. *Frontiers in Cellular Neuroscience*, 13. <https://doi.org/10.3389/fncel.2019.00133>
- Li, D., Liu, C., Yang, C., Wang, D., Wu, D., Qi, Y., Su, Q., Gao, G., Xu, Z., & Guo, Y. (2017). Slow intrathecal injection of rAAVrh10 enhances its transduction of spinal cord and therapeutic efficacy in a mutant SOD1 model of ALS. *Neuroscience*, 365, 192–205. <https://doi.org/10.1016/j.neuroscience.2017.10.001>
- Li, K., Hala, T. J., Seetharam, S., Poulsen, D. J., Wright, M. C., & Lepore, A. C. (2015). GLT1 overexpression in SOD1G93A mouse cervical spinal cord does not preserve diaphragm function or extend disease. *Neurobiology of Disease*, 78, 12–23. <https://doi.org/10.1016/j.nbd.2015.03.010>
- Li, Q., Vo, H. T., Wang, J., Fox-Quick, S., Dobrunz, L. E., & King, G. D. (2017). Klotho regulates CA1 hippocampal synaptic plasticity. *Neuroscience*, 347, 123–133. <https://doi.org/10.1016/j.neuroscience.2017.02.006>
- Li, S.-A., Watanabe, M., Yamada, H., Nagai, A., Kinuta, M., & Takei, K. (2004). Immunohistochemical Localization of Klotho Protein in Brain, Kidney, and Reproductive Organs of Mice. *Cell Structure and Function*, 29(4), 91–99. <https://doi.org/10.1247/csf.29.91>
- Liao, Y., Smyth, G. K., & Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research*, 47(8), e47–e47. <https://doi.org/10.1093/nar/gkz114>
- Lim, C. K. W., Gapinske, M., Brooks, A. K., Woods, W. S., Powell, J. E., Zeballos C., M. A., Winter, J., Perez-Pinera, P., & Gaj, T. (2020). Treatment of a Mouse Model of ALS by In Vivo Base Editing. *Molecular Therapy*, 28(4), 1177–1189. <https://doi.org/10.1016/j.jymthe.2020.01.005>
- Lin, H., Hu, H., Duan, W., Liu, Y., Tan, G., Li, Z., Liu, Y., Deng, B., Song, X., Wang, W., Wen, D., Wang, Y., & Li, C. (2018). Intramuscular Delivery of scAAV9-hlGF1 Prolongs Survival in the hSOD1G93A ALS Mouse Model via Upregulation of D-Amino Acid Oxidase. *Molecular Neurobiology*, 55(1), 682–695. <https://doi.org/10.1007/s12035-016-0335-z>
- Lin, H. Q., Hu, H. J., Duan, W. S., Liu, Y. L., Tan, G. J., Li, Z. Y., Liu, Y. K., Deng, B. Bin, Song, X. Q., Wang, W., Wen, D., Wang, Y., & Li, C. Y. (2016). Intramuscular delivery of scAAV9-hlGF1 prolongs survival in the hSOD1G93A ALS mouse model via upregulation of D-amino acid oxidase. *Molecular Neurobiology*, 55(1), 682–695. <https://doi.org/10.1007/s12035-016-0335-z>
- Lindberg, K., Amin, R., Moe, O. W., Hu, M.-C., Erben, R. G., Östman Wernerson, A., Lanske, B., Olauson, H., & Larsson, T. E. (2014). The Kidney Is the Principal Organ Mediating Klotho Effects. *Journal of the American Society of Nephrology*, 25(10), 2169–2175. <https://doi.org/10.1681/ASN.2013111209>
- Lindström, M., Chen, L., Jiang, S., Zhang, D., Gao, Y., Zheng, J., Hao, X., Yang, X., Kabbinala, A., Thoma, J., Metzger, L. C., Zhang, D. Y., Zhu, X., Liu, H., Gustafsson, C. M., Burmann, B. M., Winderickx, J., Sunnerhagen, P., & Liu, B. (2022). Lsm7 phase-separated condensates trigger stress granule formation. *Nature Communications*, 13(1), 3701. <https://doi.org/10.1038/s41467-022-31282-8>
- Ling, J. P., Pletnikova, O., Troncoso, J. C., & Wong, P. C. (2015). TDP-43 repression of nonconserved cryptic exons is compromised in ALS-FTD. *Science*, 349(6248), 650–655. <https://doi.org/10.1126/science.aab0983>
- Lino, M. M., Schneider, C., & Caroni, P. (2002). Accumulation of SOD1 Mutants in Postnatal Motoneurons Does Not Cause Motoneuron Pathology or Motoneuron Disease. *The Journal of Neuroscience*, 22(12), 4825–4832. <https://doi.org/10.1523/JNEUROSCI.22-12-04825.2002>
- Lips, M. B., & Keller, B. U. (1998). Endogenous calcium buffering in motoneurons of the nucleus hypoglossus from mouse. *The Journal of Physiology*, 511(1), 105–117. <https://doi.org/10.1111/j.1469-7793.1998.105bi.x>

- Liu, F.-T., & Hsu, D. K. (2007). The Role of Galectin-3 in Promotion of the Inflammatory Response. *Drug News & Perspectives*, 20(7), 455. <https://doi.org/10.1358/dnp.2007.20.7.1149628>
- Liu, J., Lillo, C., Andreas Jonsson, P., Vande Velde, C., Ward, C. M., Miller, T. M., Subramaniam, J. R., Rothstein, J. D., Marklund, S., Andersen, P. M., Brännström, T., Gredal, O., Wong, P. C., & Williams, D. S. (2004). Toxicity of Familial ALS-Linked SOD1 Mutants from Selective Recruitment to Spinal Mitochondria mitochondria as the basis for their selective toxicity in ALS. In *Neuron* (Vol. 43).
- Liu, Y., Andreucci, A., Iwamoto, N., Yin, Y., Yang, H., Liu, F., Bulychiev, A., Hu, X. S., Lin, X., Lamore, S., Patil, S., Mohapatra, S., Purcell-Estabrook, E., Taborn, K., Dale, E., & Vargeese, C. (2022). Preclinical evaluation of WVE-004, an investigational stereopure oligonucleotide for the treatment of C9orf72-associated ALS or FTD. *Molecular Therapy - Nucleic Acids*, 28, 558–570. <https://doi.org/10.1016/j.omtn.2022.04.007>
- Liu, Y., Pattamatta, A., Zu, T., Reid, T., Bardhi, O., Borchelt, D. R., Yachnis, A. T., & Ranum, L. P. W. (2016). C9orf72 BAC Mouse Model with Motor Deficits and Neurodegenerative Features of ALS/FTD. *Neuron*, 90(3), 521–534. <https://doi.org/10.1016/j.neuron.2016.04.005>
- Liu, Y., & Wang, H. (2020). Peripheral nerve injury induced changes in the spinal cord and strategies to counteract/enhance the changes to promote nerve regeneration. *Neural Regeneration Research*, 15(2), 189. <https://doi.org/10.4103/1673-5374.265540>
- Lobsiger, C. S., Boillee, S., McAlonis-Downes, M., Khan, A. M., Feltri, M. L., Yamanaka, K., & Cleveland, D. W. (2009). Schwann cells expressing dismutase active mutant SOD1 unexpectedly slow disease progression in ALS mice. *Proceedings of the National Academy of Sciences*, 106(11), 4465–4470. <https://doi.org/10.1073/pnas.0813339106>
- López-Erauskin, J., Tadokoro, T., Baughn, M. W., Myers, B., McAlonis-Downes, M., Chillon-Marinhas, C., Asiaban, J. N., Artates, J., Bui, A. T., Vetto, A. P., Lee, S. K., Le, A. V., Sun, Y., Jambeau, M., Boubaker, J., Swing, D., Qiu, J., Hicks, G. G., Ouyang, Z., ... Da Cruz, S. (2018). ALS/FTD-Linked Mutation in FUS Suppresses Intra-axonal Protein Synthesis and Drives Disease Without Nuclear Loss-of-Function of FUS. *Neuron*, 100(4), 816–830.e7. <https://doi.org/10.1016/j.neuron.2018.09.044>
- Lopez-Gonzalez, R., Lu, Y., Gendron, T. F., Karydas, A., Tran, H., Yang, D., Petrucelli, L., Miller, B. L., Almeida, S., & Gao, F.-B. (2016). Poly(GR) in C9ORF72 -Related ALS/FTD Compromises Mitochondrial Function and Increases Oxidative Stress and DNA Damage in iPSC-Derived Motor Neurons. *Neuron*, 92(2), 383–391. <https://doi.org/10.1016/j.neuron.2016.09.015>
- Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Lu, Y.-Y., Wang, L.-J., Muramatsu, S., Ikeguchi, K., Fujimoto, K., Okada, T., Mizukami, H., Matsushita, T., Hanazono, Y., Kume, A., Nagatsu, T., Ozawa, K., & Nakano, I. (2003). Intramuscular injection of AAV-GDNF results in sustained expression of transgenic GDNF, and its delivery to spinal motoneurons by retrograde transport. *Neuroscience Research*, 45(1), 33–40. [https://doi.org/10.1016/s0168-0102\(02\)00195-5](https://doi.org/10.1016/s0168-0102(02)00195-5)
- Ludolph, A. C., Bendotti, C., Blaugrund, E., Chio, A., Greensmith, L., Loeffler, J.-P., Mead, R., Niessen, H. G., Petri, S., Pradat, P.-F., Robberecht, W., Ruegg, M., Schwalenstöcker, B., Stiller, D., van den Berg, L., Vieira, F., & von Horsten, S. (2010). Guidelines for preclinical animal research in ALS/MND: A consensus meeting. *Amyotrophic Lateral Sclerosis*, 11(1–2), 38–45. <https://doi.org/10.3109/17482960903545334>
- Lundstrom, K. (2023). Viral Vectors in Gene Therapy: Where Do We Stand in 2023? *Viruses*, 15(3), 698. <https://doi.org/10.3390/v15030698>
- Ma, X. R., Prudencio, M., Koike, Y., Vatsavayai, S. C., Kim, G., Harbinski, F., Briner, A., Rodriguez, C. M., Guo, C., Akiyama, T., Schmidt, H. B., Cummings, B. B., Wyatt, D. W., Kurylo, K., Miller, G., Mekhoubad, S., Sallee, N., Mekonnen, G., Ganser, L., ... Gitler, A. D. (2022). TDP-43 represses cryptic exon inclusion in the FTD-ALS gene UNC13A. *Nature*, 603(7899), 124–130. <https://doi.org/10.1038/s41586-022-04424-7>
- Mackenzie, I. R. A., Bigio, E. H., Ince, P. G., Geser, F., Neumann, M., Cairns, N. J., Kwong, L. K., Forman, M. S., Ravits, J., Stewart, H., Eisen, A., McClusky, L., Kretzschmar, H. A., Monoranu, C. M., Highley, J. R., Kirby, J., Siddique, T., Shaw, P. J., Lee, V. M.-Y., & Trojanowski, J. Q. (2007). Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Annals of Neurology*, 61(5), 427–434. <https://doi.org/10.1002/ana.21147>
- Madill, M., McDonagh, K., Ma, J., Vajda, A., McLoughlin, P., O'Brien, T., Hardiman, O., & Shen, S. (2017). Amyotrophic lateral sclerosis patient iPSC-derived astrocytes impair autophagy via non-cell autonomous mechanisms. *Molecular Brain*, 10(1), 22. <https://doi.org/10.1186/s13041-017-0300-4>

- Madison, R., Sofroniew, M. V., & Robinson, G. A. (2011). Schwann cell influence on motor neuron regeneration accuracy Roger. *Bone*, 23(1), 1–7. <https://doi.org/10.1161/CIRCULATIONAHA.110.956839>
- Madji Hounoum, B., Yourc'h, P., Felix, R., Corcia, P., Patin, F., Guéguinou, M., Potier-Cartereau, M., Vandier, C., Raoul, C., Andres, C. R., Mavel, S., & Blasco, H. (2016). NSC-34 Motor Neuron-Like Cells Are Unsuitable as Experimental Model for Glutamate-Mediated Excitotoxicity. *Frontiers in Cellular Neuroscience*, 10. <https://doi.org/10.3389/fncel.2016.00118>
- Maehlen, J., Schröder, H. D., Klareskog, L., Olsson, T., & Kristensson, K. (1988). Axotomy induces MHC class I antigen expression on rat nerve cells. *Neuroscience Letters*, 92(1), 8–13. [https://doi.org/10.1016/0304-3940\(88\)90733-1](https://doi.org/10.1016/0304-3940(88)90733-1)
- Maekawa, Y., Ishikawa, K., Yasuda, O., Oguro, R., Hanasaki, H., Kida, I., Takemura, Y., Ohishi, M., Katsuya, T., & Rakugi, H. (2009). Klotho suppresses TNF- α -induced expression of adhesion molecules in the endothelium and attenuates NF- κ B activation. *Endocrine*, 35(3), 341–346. <https://doi.org/10.1007/s12020-009-9181-3>
- Maekawa, Y., Ohishi, M., Ikushima, M., Yamamoto, K., Yasuda, O., Oguro, R., Yamamoto-Hanasaki, H., Tatara, Y., Takeya, Y., & Rakugi, H. (2011). Klotho protein diminishes endothelial apoptosis and senescence via a mitogen-activated kinase pathway. *Geriatrics & Gerontology International*, 11(4), 510–516. <https://doi.org/10.1111/j.1447-0594.2011.00699.x>
- Magrané, J., Cortez, C., Gan, W.-B., & Manfredi, G. (2014). Abnormal mitochondrial transport and morphology are common pathological denominators in SOD1 and TDP43 ALS mouse models. *Human Molecular Genetics*, 23(6), 1413–1424. <https://doi.org/10.1093/hmg/ddt528>
- Magrané, J., Sahawneh, M. A., Przedborski, S., Estévez, Á. G., & Manfredi, G. (2012). Mitochondrial Dynamics and Bioenergetic Dysfunction Is Associated with Synaptic Alterations in Mutant SOD1 Motor Neurons. *The Journal of Neuroscience*, 32(1), 229–242. <https://doi.org/10.1523/JNEUROSCI.1233-11.2012>
- Mahoney, D. J., Kaczor, J. J., Bourgeois, J., Yasuda, N., & Tarnopolsky, M. A. (2006). Oxidative stress and antioxidant enzyme upregulation in SOD1-G93A mouse skeletal muscle. *Muscle and Nerve*, 33(6), 809–816. <https://doi.org/10.1002/mus.20542>
- Maltese, G., Psefteli, P., Rizzo, B., Srivastava, S., Gnudi, L., Mann, G. E., & Siow, R. C. M. (2017). The anti-ageing hormone klotho induces Nrf2-mediated antioxidant defences in human aortic smooth muscle cells. *Journal of Cellular and Molecular Medicine*, 21(3), 621–627. <https://doi.org/10.1111/jcmm.12996>
- Manabe, Y., Nagano, I., A Gazi, M. S., Murakami, T., Shiote, M., Shoji, M., Kitagawa, H., Setoguchi, Y., Abe, K., & Gazi, S. A. (2002). Adenovirus-mediated gene transfer of glial cell line-derived neurotrophic factor prevents motor neuron loss of transgenic model mice for amyotrophic lateral sclerosis. In *Apoptosis* (Vol. 7).
- Manabe, Y., Nagano, I., Gazi, A., Murakami, T., Shiote, M., Shoji, M., Kitagawa, H., & Abe, K. (2003). Glial cell line-derived neurotrophic factor protein prevents motor neuron loss of transgenic model mice for amyotrophic lateral sclerosis. *Neurological Research*, 255, 329–334. <https://doi.org/10.1179/016164103101201193>
- Mancuso, R., Martínez-Muriana, A., Leiva, T., Gregorio, D., Ariza, L., Morell, M., Esteban-Pérez, J., García-Redondo, A., Calvo, A. C., Atencia-Cibreiro, G., Corfas, G., Osta, R., Bosch, A., & Navarro, X. (2016). Neuregulin-1 promotes functional improvement by enhancing collateral sprouting in SOD1G93A ALS mice and after partial muscle denervation. *Neurobiology of Disease*, 95, 168–178. <https://doi.org/10.1016/j.nbd.2016.07.023>
- Mancuso, R., & Navarro, X. (2015). Amyotrophic lateral sclerosis: Current perspectives from basic research to the clinic. *Progress in Neurobiology*, 133, 1–26. <https://doi.org/10.1016/j.pneurobio.2015.07.004>
- Mancuso, R., Oliván, S., Mancera, P., Pastén-Zamorano, A., Manzano, R., Casas, C., Osta, R., & Navarro, X. (2012). Effect of genetic background on onset and disease progression in the SOD1-G93A model of amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis*, 13(3), 302–310. <https://doi.org/10.3109/17482968.2012.662688>
- Mancuso, R., Osta, R., & Navarro, X. (2014). Presymptomatic electrophysiological tests predict clinical onset and survival in SOD1^{G93A} ALS mice. *Muscle & Nerve*, 50(6), 943–949. <https://doi.org/10.1002/mus.24237>
- Mancuso, R., Santos-Nogueira, E., Osta, R., & Navarro, X. (2011). Electrophysiological analysis of a murine model of motoneuron disease. *Clinical Neurophysiology*, 122(8), 1660–1670. <https://doi.org/10.1016/j.clinph.2011.01.045>
- Mansilla-Soto, J., Riviere, I., Boulad, F., & Sadelain, M. (2016). Cell and Gene Therapy for the Beta-Thalassemias: Advances and Prospects. *Human Gene Therapy*, 27(4), 295–304. <https://doi.org/10.1089/hum.2016.037>

- Manzano, R., Toivonen, J. M., Oliván, S., Calvo, A. C., Moreno-Igoa, M., Muñoz, M. J., Zaragoza, P., García-Redondo, A., & Osta, R. (2011). Altered Expression of Myogenic Regulatory Factors in the Mouse Model of Amyotrophic Lateral Sclerosis. *Neurodegenerative Diseases*, 8(5), 386–396. <https://doi.org/10.1159/000324159>
- Marcuzzo, S., Zucca, I., Mastropietro, A., de Rosbo, N. K., Cavalcante, P., Tartari, S., Bonanno, S., Preite, L., Mantegazza, R., & Bernasconi, P. (2011). Hind limb muscle atrophy precedes cerebral neuronal degeneration in G93A-SOD1 mouse model of amyotrophic lateral sclerosis: A longitudinal MRI study. *Experimental Neurology*, 231(1), 30–37. <https://doi.org/10.1016/j.expneurol.2011.05.007>
- Marinković, P., Reuter, M. S., Brill, M. S., Godinho, L., Kerschensteiner, M., & Misgeld, T. (2012). Axonal transport deficits and degeneration can evolve independently in mouse models of amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(11), 4296–4301. <https://doi.org/10.1073/pnas.1200658109>
- Martier, R., Liefhebber, J. M., García-Osta, A., Miniarikova, J., Cuadrado-Tejedor, M., Espelosin, M., Ursua, S., Petry, H., van Deventer, S. J., Evers, M. M., & Konstantinova, P. (2019). Targeting RNA-Mediated Toxicity in C9orf72 ALS and/or FTD by RNAi-Based Gene Therapy. *Molecular Therapy - Nucleic Acids*, 16(June), 26–37. <https://doi.org/10.1016/j.omtn.2019.02.001>
- Martin, L. J. (1999). Neuronal Death in Amyotrophic Lateral Sclerosis Is Apoptosis: Possible Contribution of a Programmed Cell Death Mechanism. *Journal of Neuropathology and Experimental Neurology*, 58(5), 459–471. <https://doi.org/10.1097/00005072-199905000-00005>
- Martineau, É., Arbour, D., Vallée, J., & Robitaille, R. (2020). Properties of Glial Cell at the Neuromuscular Junction Are Incompatible with Synaptic Repair in the SOD1^{G37R} ALS Mouse Model. *The Journal of Neuroscience*, 40(40), 7759–7777. <https://doi.org/10.1523/JNEUROSCI.1748-18.2020>
- Masrori, P., & Van Damme, P. (2020). Amyotrophic lateral sclerosis: a clinical review. In *European Journal of Neurology* (Vol. 27, Issue 10, pp. 1918–1929). Blackwell Publishing Ltd. <https://doi.org/10.1111/ene.14393>
- Massó, A., Sánchez, A., Bosch, A., Giménez-Llort, L., & Chillón, M. (2018). Secreted α Klotho isoform protects against age-dependent memory deficits. *Molecular Psychiatry*, 23(9), 1937–1947. <https://doi.org/10.1038/mp.2017.211>
- Massó, A., Sánchez, A., Gimenez-Llort, L., Lizcano, J. M., Cañete, M., Garcá, B., Torres-Lista, V., Puig, M., Bosch, A., & Chillon, M. (2015). Secreted and transmembrane α klotho isoforms have different spatio-temporal profiles in the brain during aging and Alzheimer's disease progression. *PLoS ONE*, 10(11), 1–15. <https://doi.org/10.1371/journal.pone.0143623>
- Masuda, H., Chikuda, H., Suga, T., Kawaguchi, H., & Kuro-o, M. (2005). Regulation of multiple ageing-like phenotypes by inducible klotho gene expression in klotho mutant mice. *Mechanisms of Ageing and Development*, 126(12), 1274–1283. <https://doi.org/10.1016/j.mad.2005.07.007>
- Matsumura, Y., Aizawa, H., Shiraki-Iida, T., Nagai, R., Kuro-o, M., & Nabeshima, Y. (1998). Identification of the HumanKlothoGene and Its Two Transcripts Encoding Membrane and SecretedKlothoProtein. *Biochemical and Biophysical Research Communications*, 242(3), 626–630. <https://doi.org/10.1006/bbrc.1997.8019>
- Mauro, V. P., & Chappell, S. A. (2014). A critical analysis of codon optimization in human therapeutics. *Trends in Molecular Medicine*, 20(11), 604–613. <https://doi.org/10.1016/j.molmed.2014.09.003>
- McCampbell, A., Cole, T., Wegener, A. J., Tomassy, G. S., Setnicka, A., Farley, B. J., Schoch, K. M., Hoyer, M. L., Shabsovich, M., Sun, L., Luo, Y., Zhang, M., Thankamony, S., Salzman, D. W., Cudkowicz, M., Graham, D. L., Bennett, C. F., Kordasiewicz, H. B., Swayze, E. E., & Miller, T. M. (2018). Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models. *Journal of Clinical Investigation*, 128(8), 3558–3567. <https://doi.org/10.1172/JCI99081>
- McCombe, P. A., & Henderson, R. D. (2010). Effects of gender in amyotrophic lateral sclerosis. *Gender Medicine*, 7(6), 557–570. <https://doi.org/10.1016/j.genm.2010.11.010>
- Mead, R. J., Bennett, E. J., Kennerley, A. J., Sharp, P., Sunyach, C., Kasher, P., Berwick, J., Pettmann, B., Battaglia, G., Azzouz, M., Grierson, A., & Shaw, P. J. (2011). Optimised and Rapid Pre-clinical Screening in the SOD1G93A Transgenic Mouse Model of Amyotrophic Lateral Sclerosis (ALS). *PLoS ONE*, 6(8), e23244. <https://doi.org/10.1371/journal.pone.0023244>
- Mead, R. J., Shan, N., Reiser, H. J., Marshall, F., & Shaw, P. J. (2023). Amyotrophic lateral sclerosis: a neurodegenerative disorder poised for successful therapeutic translation. In *Nature Reviews Drug Discovery* (Vol. 22, Issue 3, pp. 185–212). Nature Research. <https://doi.org/10.1038/s41573-022-00612-2>

- Mencke, R., Harms, G., Moser, J., van Meurs, M., Diepstra, A., Leuvenink, H. G., & Hillebrands, J.-L. (2017). Human alternative Klotho mRNA is a nonsense-mediated mRNA decay target inefficiently spliced in renal disease. *JCI Insight*, 2(20). <https://doi.org/10.1172/jci.insight.94375>
- Merk, M., Mitchell, R. A., Endres, S., & Bucala, R. (2012). D-dopachrome tautomerase (D-DT or MIF-2): Doubling the MIF cytokine family. *Cytokine*, 59(1), 10–17. <https://doi.org/10.1016/j.cyto.2012.03.014>
- Merk, M., Zierow, S., Leng, L., Das, R., Du, X., Schulte, W., Fan, J., Lue, H., Chen, Y., Xiong, H., Chagnon, F., Bernhagen, J., Lolis, E., Mor, G., Lesur, O., & Bucala, R. (2011). The D -dopachrome tautomerase (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF). *Proceedings of the National Academy of Sciences*, 108(34). <https://doi.org/10.1073/pnas.1102941108>
- Mietzsch, M., Broecker, F., Reinhardt, A., Seeberger, P. H., & Heilbronn, R. (2014). Differential Adeno-Associated Virus Serotype-Specific Interaction Patterns with Synthetic Heparins and Other Glycans. *Journal of Virology*, 88(5), 2991–3003. <https://doi.org/10.1128/JVI.03371-13>
- Miller, T., Cudkowicz, M., Shaw, P. J., Andersen, P. M., Atassi, N., Bucelli, R. C., Genge, A., Glass, J., Ladha, S., Ludolph, A. L., Maragakis, N. J., McDermott, C. J., Pestronk, A., Ravits, J., Salachas, F., Trudell, R., Van Damme, P., Zinman, L., Bennett, C. F., ... Ferguson, T. A. (2020). Phase 1–2 Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *New England Journal of Medicine*, 383(2), 109–119. <https://doi.org/10.1056/NEJMoa2003715>
- Miller, T. M., Cudkowicz, M. E., Genge, A., Shaw, P. J., Sobue, G., Bucelli, R. C., Chiò, A., Van Damme, P., Ludolph, A. C., Glass, J. D., Andrews, J. A., Babu, S., Benatar, M., McDermott, C. J., Cochrane, T., Chary, S., Chew, S., Zhu, H., Wu, F., ... Fradette, S. (2022). Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *New England Journal of Medicine*, 387(12), 1099–1110. <https://doi.org/10.1056/NEJMoa2204705>
- Miller, T. M., Kaspar, B. K., Kops, G. J., Yamanaka, K., Christian, L. J., Gage, F. H., & Cleveland, D. W. (2005). Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. *Annals of Neurology*, 57(5), 773–776. <https://doi.org/10.1002/ana.20453>
- Miller, T. M., Kim, S. H., Yamanaka, K., Hester, M., Umapathi, P., Arnson, H., Rizo, L., Mendell, J. R., Gage, F. H., Cleveland, D. W., & Kaspar, B. K. (2006). Gene transfer demonstrates that muscle is not a primary target for non-cell-autonomous toxicity in familial amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences*, 103(51), 19546–19551. <https://doi.org/10.1073/pnas.0609411103>
- Miller, T. M., Pestronk, A., David, W., Rothstein, J., Simpson, E., Appel, S. H., Andres, P. L., Mahoney, K., Allred, P., Alexander, K., Ostrow, L. W., Schoenfeld, D., Macklin, E. A., Norris, D. A., Manousakis, G., Crisp, M., Smith, R., Bennett, C. F., Bishop, K. M., & Cudkowicz, M. E. (2013). An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *The Lancet Neurology*, 12(5), 435–442. [https://doi.org/10.1016/S1474-4422\(13\)70061-9](https://doi.org/10.1016/S1474-4422(13)70061-9)
- Mimoto, T., Miyazaki, K., Morimoto, N., Kurata, T., Satoh, K., Ikeda, Y., & Abe, K. (2012). Impaired antioxidant Keap1/Nrf2 system and the downstream stress protein responses in the motor neuron of ALS model mice. *Brain Research*, 1446, 109–118. <https://doi.org/10.1016/j.brainres.2011.12.064>
- Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J., & Bucala, R. (2002). Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: Regulatory role in the innate immune response. *Proceedings of the National Academy of Sciences*, 99(1), 345–350. <https://doi.org/10.1073/pnas.012511599>
- Miyoshi, S., Tezuka, T., Arimura, S., Tomono, T., Okada, T., & Yamanashi, Y. (2017). DOK7 gene therapy enhances motor activity and life span in ALS model mice. *EMBO Molecular Medicine*, 9(7), 880–889. <https://doi.org/10.15252/emmm.201607298>
- Mòdol, L., Mancuso, R., Alé, A., Francos-Quijorna, I., & Navarro, X. (2014). Differential effects on KCC2 expression and spasticity of ALS and traumatic injuries to motoneurons. *Frontiers in Cellular Neuroscience*, 8. <https://doi.org/10.3389/fncel.2014.00007>
- Mòdol-Caballero, G., García-Lareu, B., Herrando-Grabulosa, M., Verdés, S., López-Vales, R., Pagès, G., Chillón, M., Navarro, X., & Bosch, A. (2021a). Specific Expression of Glial-Derived Neurotrophic Factor in Muscles as Gene Therapy Strategy for Amyotrophic Lateral Sclerosis. *Neurotherapeutics*, 18(2), 1113–1126. <https://doi.org/10.1007/s13311-021-01025-6>
- Mòdol-Caballero, G., García-Lareu, B., Verdés, S., Ariza, L., Sánchez-Brualla, I., Brocard, F., Bosch, A., Navarro, X., & Herrando-Grabulosa, M. (2020a). Therapeutic Role of Neuregulin 1 Type III in SOD1-Linked Amyotrophic Lateral Sclerosis. *Neurotherapeutics*. <https://doi.org/10.1007/s13311-019-00811-7>
- Mòdol-Caballero, G., Herrando-Grabulosa, M., García-Lareu, B., Solanes, N., Verdés, S., Osta, R., Francos-Quijorna, I., López-Vales, R., Calvo, A. C., Bosch, A., & Navarro, X. (2020b). Gene therapy for

- overexpressing Neuregulin 1 type I in skeletal muscles promotes functional improvement in the SOD1G939 ALS mice. *Neurobiology of Disease*, 137. <https://doi.org/10.1016/j.nbd.2020.104793>
- Mòdol-Caballero, G., Herrando-Grabulosa, M., Verdés, S., García-Lareu, B., Hernández, N., Francos-Quijorna, I., López-Vales, R., Bosch, A., & Navarro, X. (2021b). Gene Therapy Overexpressing Neuregulin 1 Type I in Combination With Neuregulin 1 Type III Promotes Functional Improvement in the SOD1G93A ALS Mice. *Frontiers in Neurology*, 12. <https://doi.org/10.3389/fneur.2021.693309>
- Mohajeri, M. H., Figlewicz, D. A., & Bohn, M. C. (1999). Intramuscular Grafts of Myoblasts Genetically Modified to Secrete Glial Cell Line-Derived Neurotrophic Factor Prevent Motoneuron Loss and Disease Progression in a Mouse Model of Familial Amyotrophic Lateral Sclerosis. In *HUMAN GENE THERAPY* (Vol. 10). Mary Ann Liebert, Inc.
- Mori, K., Weng, S.-M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., Schmid, B., Kretschmar, H. A., Cruts, M., Van Broeckhoven, C., Haass, C., & Edbauer, D. (2013). The C9orf72 GGGGCC Repeat Is Translated into Aggregating Dipeptide-Repeat Proteins in FTL/ALS. *Science*, 339(6125), 1335–1338. <https://doi.org/10.1126/science.1232927>
- Mou, T., Deng, W., Gu, F., Pawitan, Y., & Vu, T. N. (2020). Reproducibility of Methods to Detect Differentially Expressed Genes from Single-Cell RNA Sequencing. *Frontiers in Genetics*, 10. <https://doi.org/10.3389/fgene.2019.01331>
- Mytych, J., Romerowicz-Misielak, M., & Kozirowski, M. (2018). Klotho protects human monocytes from LPS-induced immune impairment associated with immunosenescent-like phenotype. *Molecular and Cellular Endocrinology*, 470, 1–13. <https://doi.org/10.1016/j.mce.2017.05.003>
- Mytych, J., Solek, P., & Kozirowski, M. (2019). Klotho modulates ER-mediated signaling crosstalk between prosurvival autophagy and apoptotic cell death during LPS challenge. *Apoptosis*, 24(1–2), 95–107. <https://doi.org/10.1007/s10495-018-1496-1>
- Nabeshima, Y., & Imura, H. (2008). α -Klotho: A Regulator That Integrates Calcium Homeostasis. *American Journal of Nephrology*, 28(3), 455–464. <https://doi.org/10.1159/000112824>
- Nagai, M., Aoki, M., Miyoshi, I., Kato, M., Pasinelli, P., Kasai, N., Brown, R. H., & Itoyama, Y. (2001). Rats Expressing Human Cytosolic Copper–Zinc Superoxide Dismutase Transgenes with Amyotrophic Lateral Sclerosis: Associated Mutations Develop Motor Neuron Disease. *The Journal of Neuroscience*, 21(23), 9246–9254. <https://doi.org/10.1523/JNEUROSCI.21-23-09246.2001>
- Nagai, M., Re, D. B., Nagata, T., Chalazonitis, A., Jessell, T. M., Wichterle, H., & Przedborski, S. (2007). Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nature Neuroscience*, 10(5), 615–622. <https://doi.org/10.1038/nn1876>
- Nakai, H., Storm, T. A., & Kay, M. A. (2000). Increasing the size of rAAV-mediated expression cassettes in vivo by intermolecular joining of two complementary vectors. *Nature Biotechnology*, 18(5), 527–532. <https://doi.org/10.1038/75390>
- Nakata, M., Kuwabara, S., Kanai, K., Misawa, S., Tamura, N., Sawai, S., Hattori, T., & Bostock, H. (2006). Distal excitability changes in motor axons in amyotrophic lateral sclerosis. *Clinical Neurophysiology*, 117(7), 1444–1448. <https://doi.org/10.1016/j.clinph.2006.04.005>
- Naldini, L. (2015). Gene therapy returns to centre stage. *Nature*, 526(7573), 351–360. <https://doi.org/10.1038/nature15818>
- Nanou, A., Higginbottom, A., Valori, C. F., Wyles, M., Ning, K., Shaw, P., & Azzouz, M. (2013). Viral delivery of antioxidant genes as a therapeutic strategy in experimental models of amyotrophic lateral sclerosis. *Molecular Therapy*, 21(8), 1486–1496. <https://doi.org/10.1038/mt.2013.115>
- Nardo, G., Iennaco, R., Fusi, N., Heath, P. R., Marino, M., Trolese, M. C., Ferraiuolo, L., Lawrence, N., Shaw, P. J., & Bendotti, C. (2013). Transcriptomic indices of fast and slow disease progression in two mouse models of amyotrophic lateral sclerosis. *Brain*, 136(11), 3305–3332. <https://doi.org/10.1093/brain/awt250>
- Navarro, X., & Udina, E. (2009). Chapter 6 *Methods and Protocols in Peripheral Nerve Regeneration Experimental Research* (pp. 105–126). [https://doi.org/10.1016/S0074-7742\(09\)87006-2](https://doi.org/10.1016/S0074-7742(09)87006-2)
- Neubrand, V. E., Thomas, C., Schmidt, S., Debant, A., & Schiavo, G. (2010). Kidins220/ARMS regulates Rac1-dependent neurite outgrowth by direct interaction with the RhoGEF Trio. *Journal of Cell Science*, 123(12), 2111–2123. <https://doi.org/10.1242/jcs.064055>
- Neumann, H., Cavalié, A., Jenne, D. E., & Wekerle, H. (1995). Induction of MHC Class I Genes in Neurons. *Science*, 269(5223), 549–552. <https://doi.org/10.1126/science.7624779>

- Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., McCluskey, L. F., Miller, B. L., Masliah, E., Mackenzie, I. R., Feldman, H., Feiden, W., Kretzschmar, H. A., Trojanowski, J. Q., & Lee, V. M. Y. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*, 314(5796), 130–133. <https://doi.org/10.1126/science.1134108>
- Neymotin, A., Calingasan, N. Y., Wille, E., Naseri, N., Petri, S., Damiano, M., Liby, K. T., Risingsong, R., Sporn, M., Beal, M. F., & Kiaei, M. (2011). Neuroprotective effect of Nrf2/ARE activators, CDDO ethylamide and CDDO trifluoroethylamide, in a mouse model of amyotrophic lateral sclerosis. *Free Radical Biology and Medicine*, 51(1), 88–96. <https://doi.org/10.1016/j.freeradbiomed.2011.03.027>
- Nihei, K., McKee, A. C., & Kowall, N. W. (1993). Patterns of neuronal degeneration in the motor cortex of amyotrophic lateral sclerosis patients. *Acta Neuropathologica*, 86(1), 55–64. <https://doi.org/10.1007/BF00454899>
- Nizzardo, M., Taiana, M., Rizzo, F., Aguila Benitez, J., Nijssen, J., Allodi, I., Melzi, V., Bresolin, N., Comi, G. P., Hedlund, E., & Corti, S. (2020). Synaptotagmin 13 is neuroprotective across motor neuron diseases. *Acta Neuropathologica*, 139(5), 837–853. <https://doi.org/10.1007/s00401-020-02133-x>
- Oguro, A., & Imaoka, S. (2019). Thioredoxin-related transmembrane protein 2 (TMX2) regulates the Ran protein gradient and importin- β -dependent nuclear cargo transport. *Scientific Reports*, 9(1), 15296. <https://doi.org/10.1038/s41598-019-51773-x>
- Ohsawa, Y., Ohtsubo, H., Mune Kane, A., Ohkubo, K., Murakami, T., Fujino, M., Nishimatsu, S., Hagiwara, H., Nishimura, H., Kaneko, R., Suzuki, T., Tatsumi, R., Mizunoya, W., Hinohara, A., Fukunaga, M., & Sunada, Y. (2023). Circulating α -Klotho Counteracts Transforming Growth Factor- β -Induced Sarcopenia. *The American Journal of Pathology*, 193(5), 591–607. <https://doi.org/10.1016/j.ajpath.2023.01.009>
- Okamoto, K. (2008). Bunina bodies in amyotrophic lateral sclerosis. *Neuropathology*, 28(2), 109.
- Olauson, H., Lindberg, K., Amin, R., Jia, T., Wernerson, A., Andersson, G., & Larsson, T. E. (2012). Targeted Deletion of Klotho in Kidney Distal Tubule Disrupts Mineral Metabolism. *Journal of the American Society of Nephrology*, 23(10), 1641–1651. <https://doi.org/10.1681/ASN.2012010048>
- O'Rourke, J. G., Bogdanik, L., Muhammad, A. K. M. G., Gendron, T. F., Kim, K. J., Austin, A., Cady, J., Liu, E. Y., Zarrow, J., Grant, S., Ho, R., Bell, S., Carmona, S., Simpkinson, M., Lall, D., Wu, K., Daugherty, L., Dickson, D. W., Harms, M. B., ... Baloh, R. H. (2015). C9orf72 BAC Transgenic Mice Display Typical Pathologic Features of ALS/FTD. *Neuron*, 88(5), 892–901. <https://doi.org/10.1016/j.neuron.2015.10.027>
- O'Rourke, J. G., Bogdanik, L., Yáñez, A., Lall, D., Wolf, A. J., Muhammad, A. K. M. G., Ho, R., Carmona, S., Vit, J. P., Zarrow, J., Kim, K. J., Bell, S., Harms, M. B., Miller, T. M., Dangler, C. A., Underhill, D. M., Goodridge, H. S., Lutz, C. M., & Baloh, R. H. (2016). C9orf72 is required for proper macrophage and microglial function in mice. *Science*, 351(6279), 1324–1329. <https://doi.org/10.1126/science.aaf1064>
- Pacak, C. A., Sakai, Y., Thattaliyath, B. D., Mah, C. S., & Byrne, B. J. (2008). Tissue specific promoters improve specificity of AAV9 mediated transgene expression following intra-vascular gene delivery in neonatal mice. *Genetic Vaccines and Therapy*, 6(1), 13. <https://doi.org/10.1186/1479-0556-6-13>
- Paganoni, S., Hendrix, S., Dickson, S. P., Knowlton, N., Macklin, E. A., Berry, J. D., Elliott, M. A., Maise, S., Karam, C., Caress, J. B., Owegi, M. A., Quick, A., Wymer, J., Goutman, S. A., Heitzman, D., Heiman-Patterson, T. D., Jackson, C. E., Quinn, C., Rothstein, J. D., ... Cudkowicz, M. E. (2021). Long-term survival of participants in the <scp>CENTAUR</scp> trial of sodium phenylbutyrate-taurursodiol in <scp>amyotrophic lateral sclerosis</scp>. *Muscle & Nerve*, 63(1), 31–39. <https://doi.org/10.1002/mus.27091>
- Paganoni, S., Macklin, E. A., Hendrix, S., Berry, J. D., Elliott, M. A., Maise, S., Karam, C., Caress, J. B., Owegi, M. A., Quick, A., Wymer, J., Goutman, S. A., Heitzman, D., Heiman-Patterson, T., Jackson, C. E., Quinn, C., Rothstein, J. D., Kasarskis, E. J., Katz, J., ... Cudkowicz, M. E. (2020). Trial of Sodium Phenylbutyrate–Taurursodiol for Amyotrophic Lateral Sclerosis. *New England Journal of Medicine*, 383(10), 919–930. <https://doi.org/10.1056/NEJMoa1916945>
- Pagès, G., Giménez-Llort, L., García-Lareu, B., Ariza, L., Navarro, M., Casas, C., Chillón, M., & Bosch, A. (2019). Intrathecal AAVrh10 corrects biochemical and histological hallmarks of mucopolysaccharidosis VII mice and improves behavior and survival. *Human Molecular Genetics*, 28(21), 3610–3624. <https://doi.org/10.1093/hmg/ddz220>
- Pan, S., Chen, R., Aebersold, R., & Brentnall, T. A. (2011). Mass Spectrometry Based Glycoproteomics—From a Proteomics Perspective. *Molecular & Cellular Proteomics*, 10(1), R110.003251. <https://doi.org/10.1074/mcp.R110.003251>

- Pang, Z. P., Melicoff, E., Padgett, D., Liu, Y., Teich, A. F., Dickey, B. F., Lin, W., Adachi, R., & Südhof, T. C. (2006). Synaptotagmin-2 Is Essential for Survival and Contributes to Ca^{2+} Triggering of Neurotransmitter Release in Central and Neuromuscular Synapses. *The Journal of Neuroscience*, 26(52), 13493–13504. <https://doi.org/10.1523/JNEUROSCI.3519-06.2006>
- Park, S.-J., Shin, E.-J., Min, S. S., An, J., Li, Z., Hee Chung, Y., Hoon Jeong, J., Bach, J.-H., Nah, S.-Y., Kim, W.-K., Jang, C.-G., Kim, Y.-S., Nabeshima, Y., Nabeshima, T., & Kim, H.-C. (2013). Inactivation of JAK2/STAT3 Signaling Axis and Downregulation of M1 mAChR Cause Cognitive Impairment in klotho Mutant Mice, a Genetic Model of Aging. *Neuropsychopharmacology*, 38(8), 1426–1437. <https://doi.org/10.1038/npp.2013.39>
- Parone, P. A., Da Cruz, S., Han, J. S., McAlonis-Downes, M., Vetto, A. P., Lee, S. K., Tseng, E., & Cleveland, D. W. (2013). Enhancing mitochondrial calcium buffering capacity reduces aggregation of misfolded SOD1 and motor neuron cell death without extending survival in mouse models of inherited amyotrophic lateral sclerosis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 33(11), 4657–4671. <https://doi.org/10.1523/JNEUROSCI.1119-12.2013>
- Pasinelli, P., Belford, M. E., Lennon, N., Bacsikai, B. J., Hyman, B. T., Trotti, D., & Brown, R. H. (2004). Amyotrophic Lateral Sclerosis-Associated SOD1 Mutant Proteins Bind and Aggregate with Bcl-2 in Spinal Cord Mitochondria. In *Neuron* (Vol. 43).
- Pasterkamp, R. J., Giger, R. J., Baker, R. E., Hermens, W. T. J. M. C., & Verhaagen, J. (2000). Ectopic adenoviral vector-directed expression of Sema3A in organotypic spinal cord explants inhibits growth of primary sensory afferents. *Developmental Biology*, 220(2), 129–141. <https://doi.org/10.1006/dbio.2000.9627>
- Patel, P., Kriz, J., Gravel, M., Soucy, G., Bareil, C., Gravel, C., & Julien, J. P. (2014). Adeno-associated virus-mediated delivery of a recombinant single-chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis. *Molecular Therapy*, 22(3), 498–510. <https://doi.org/10.1038/mt.2013.239>
- Pavlatou, M. G., Remaley, A. T., & Gold, P. W. (2016). Klotho: a humeral mediator in CSF and plasma that influences longevity and susceptibility to multiple complex disorders, including depression. *Translational Psychiatry*, 6(8), e876–e876. <https://doi.org/10.1038/tp.2016.135>
- Paxinos, G., & Franklin, K. B. J. (2004). *The Mouse Brain in Stereotaxic Coordinates* (2nd ed.). Elsevier Science.
- Pedersen, L., Pedersen, S. M., Brasen, C. L., & Rasmussen, L. M. (2013). Soluble serum Klotho levels in healthy subjects. Comparison of two different immunoassays. *Clinical Biochemistry*, 46(12), 1079–1083. <https://doi.org/10.1016/j.clinbiochem.2013.05.046>
- Pereira, J. D., DuBreuil, D. M., Devlin, A.-C., Held, A., Sapir, Y., Berezovski, E., Hawrot, J., Dorfman, K., Chander, V., & Wainger, B. J. (2021). Human sensorimotor organoids derived from healthy and amyotrophic lateral sclerosis stem cells form neuromuscular junctions. *Nature Communications*, 12(1), 4744. <https://doi.org/10.1038/s41467-021-24776-4>
- Perry, T. L. (1990). Amyotrophic lateral sclerosis: amino acid levels in plasma and cerebrospinal fluid. *Annals of Neurology*, 28(1), 12.
- Petrozziello, T., Secondo, A., Tedeschi, V., Esposito, A., Sisalli, M., Scorziello, A., Di Renzo, G., & Annunziato, L. (2017). ApoSOD1 lacking dismutase activity neuroprotects motor neurons exposed to beta-methylamino-L-alanine through the Ca^{2+} /Akt/ERK1/2 prosurvival pathway. *Cell Death & Differentiation*, 24(3), 511–522. <https://doi.org/10.1038/cdd.2016.154>
- Pfohl, S. R., Halicek, M. T., & Mitchell, C. S. (2015). Characterization of the Contribution of Genetic Background and Gender to Disease Progression in the SOD1 G93A Mouse Model of Amyotrophic Lateral Sclerosis: A Meta-Analysis. *Journal of Neuromuscular Diseases*, 2(2), 137–150. <https://doi.org/10.3233/JND-140068>
- Piedra, J., Ontiveros, M., Miravet, S., Penalva, C., Monfar, M., & Chillon, M. (2015). Development of a Rapid, Robust, and Universal PicoGreen-Based Method to Titer Adeno-Associated Vectors. *Human Gene Therapy Methods*, 26(1), 35–42. <https://doi.org/10.1089/hgtb.2014.120>
- Pivetta, E., Capuano, A., Vescovo, M., Scanziani, E., Cappelleri, A., Rampioni Vinciguerra, G. L., Vecchione, A., Doliana, R., Mongiat, M., & Spessotto, P. (2022). EMILIN-1 deficiency promotes chronic inflammatory disease through TGF β signaling alteration and impairment of the $\text{gC1q}/\alpha4\beta1$ integrin interaction. *Matrix Biology*, 111, 133–152. <https://doi.org/10.1016/j.matbio.2022.06.005>
- Powell, J. E., Lim, C. K. W., Krishnan, R., McCallister, T. X., Saporito-Magriña, C., Zeballos, M. A., McPheron, G. D., & Gaj, T. (2022). Targeted gene silencing in the nervous system with CRISPR-Cas13. *Science Advances*, 8(3). <https://doi.org/10.1126/sciadv.abk2485>

- Pramatarova, A., Laganière, J., Roussel, J., Brisebois, K., & Rouleau, G. A. (2001). Neuron-Specific Expression of Mutant Superoxide Dismutase 1 in Transgenic Mice Does Not Lead to Motor Impairment. *The Journal of Neuroscience*, 21(10), 3369–3374. <https://doi.org/10.1523/JNEUROSCI.21-10-03369.2001>
- Proudfoot, N. (1991). Poly(A) signals. *Cell*, 64(4), 671–674. [https://doi.org/10.1016/0092-8674\(91\)90495-K](https://doi.org/10.1016/0092-8674(91)90495-K)
- Prud'homme, G. J., Kurt, M., & Wang, Q. (2022). Pathobiology of the Klotho Antiaging Protein and Therapeutic Considerations. *Frontiers in Aging*, 3. <https://doi.org/10.3389/fragi.2022.931331>
- Pupo, A., Fernández, A., Low, S. H., François, A., Suárez-Amarán, L., & Samulski, R. J. (2022). AAV vectors: The Rubik's cube of human gene therapy. *Molecular Therapy*, 30(12), 3515–3541. <https://doi.org/10.1016/j.ymthe.2022.09.015>
- Quintão, N. L. M., Balz, D., Santos, A. R. S., Campos, M. M., & Calixto, J. B. (2006). Long-lasting neuropathic pain induced by brachial plexus injury in mice: Role triggered by the pro-inflammatory cytokine, tumour necrosis factor α . *Neuropharmacology*, 50(5), 614–620. <https://doi.org/10.1016/j.neuropharm.2005.11.007>
- Ralph, G. S., Radcliffe, P. A., Day, D. M., Carthy, J. M., Leroux, M. A., Lee, D. C. P., Wong, L.-F., Bilsland, L. G., Greensmith, L., Kingsman, S. M., Mitrophanous, K. A., Mazarakis, N. D., & Azzouz, M. (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nature Medicine*, 11(4), 429–433. <https://doi.org/10.1038/nm1205>
- Rao, S. D., Yin, H. Z., & Weiss, J. H. (2003). Disruption of Glial Glutamate Transport by Reactive Oxygen Species Produced in Motor Neurons. *The Journal of Neuroscience*, 23(7), 2627–2633. <https://doi.org/10.1523/JNEUROSCI.23-07-02627.2003>
- Rao, Z., Landry, T., Li, P., Bunner, W., Laing, B. T., Yuan, Y., & Huang, H. (2019). Administration of alpha klotho reduces liver and adipose lipid accumulation in obese mice. *Heliyon*, 5(4), e01494. <https://doi.org/10.1016/j.heliyon.2019.e01494>
- Raoul, C., Abbas-Terki, T., Bensadoun, J. C., Guillot, S., Haase, G., Szulc, J., Henderson, C. E., & Aebischer, P. (2005). Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nature Medicine*, 11(4), 423–428. <https://doi.org/10.1038/nm1207>
- Ratti, A., & Buratti, E. (2016). Physiological functions and pathobiology of TDP-43 and FUS/TLS proteins. *Journal of Neurochemistry*, 138, 95–111. <https://doi.org/10.1111/jnc.13625>
- Reaume, A. G., Elliott, J. L., Hoffman, E. K., Kowa, N. W., Ferrante, R. J., Siwek, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Jr, R. H. B., Scott, R. W., Snider, W. D., & Chester, W. (1996). *Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury*. 13(may), 43–47.
- Reddy, K., Zamiri, B., Stanley, S. Y. R., Macgregor, R. B., & Pearson, C. E. (2013). The Disease-associated r(GGGGCC) Repeat from the C9orf72 Gene Forms Tract Length-dependent Uni- and Multimolecular RNA G-quadruplex Structures. *Journal of Biological Chemistry*, 288(14), 9860–9866. <https://doi.org/10.1074/jbc.C113.452532>
- Renaud-Gabardos, E. (2015). Internal ribosome entry site-based vectors for combined gene therapy. *World Journal of Experimental Medicine*, 5(1), 11. <https://doi.org/10.5493/wjem.v5.i1.11>
- Renton, A. E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J. R., Schymick, J. C., Laaksovirta, H., van Swieten, J. C., Myllykangas, L., Kalimo, H., Paetau, A., Abramzon, Y., Remes, A. M., Kaganovich, A., Scholz, S. W., Duckworth, J., Ding, J., Harmer, D. W., ... Traynor, B. J. (2011). A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD. *Neuron*, 72(2), 257–268. <https://doi.org/10.1016/j.neuron.2011.09.010>
- Ridoux, V., Robert, J.-J., Perricaudet, M., Mallet, J., Le Gal, G., & Salle, L. (1995). Adenovirus mediated gene transfer in organotypic brain slices. In *Neurobiology of Disease* (Vol. 2).
- Riemsлагh, F. W., van der Toorn, E. C., Verhagen, R. F. M., Maas, A., Bosman, L. W. J., Hukema, R. K., & Willemsen, R. (2021). Inducible expression of human C9ORF72 36× G4C2 hexanucleotide repeats is sufficient to cause RAN translation and rapid muscular atrophy in mice. *Disease Models & Mechanisms*, 14(2). <https://doi.org/10.1242/dmm.044842>
- Ripps, M. E., Huntley, G. W., Hof, P. R., Morrison, J. H., & Gordon, J. W. (1995). Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences*, 92(3), 689–693. <https://doi.org/10.1073/pnas.92.3.689>

- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, 43(7), e47–e47. <https://doi.org/10.1093/nar/gkv007>
- Riva, N., Gentile, F., Cerri, F., Gallia, F., Podini, P., Dina, G., Falzone, Y. M., Fazio, R., Lunetta, C., Calvo, A., Logroscino, G., Lauria, G., Corbo, M., Iannaccone, S., Chiò, A., Lazzerini, A., Nobile-Orazio, E., Filippi, M., & Quattrini, A. (2022). Phosphorylated TDP-43 aggregates in peripheral motor nerves of patients with amyotrophic lateral sclerosis. *Brain*, 145(1), 276–284. <https://doi.org/10.1093/brain/awab285>
- Rochat, C., Bernard-Marissal, N., Källstig, E., Pradervand, S., Perrin, F. E., Aebischer, P., Raoul, C., & Schneider, B. L. (2022). Astrocyte-targeting RNA interference against mutated superoxide dismutase 1 induces motoneuron plasticity and protects fast-fatigable motor units in a mouse model of amyotrophic lateral sclerosis. *GLIA*, 70(5), 842–857. <https://doi.org/10.1002/glia.24140>
- Roig-Soriano, J., Griñán-Ferré, C., Espinosa-Parrilla, J. F., Abraham, C. R., Bosch, A., Pallàs, M., & Chillón, M. (2022). AAV-mediated expression of secreted and transmembrane α Klotho isoforms rescues relevant aging hallmarks in senescent SAMP8 mice. *Aging Cell*, 21(4). <https://doi.org/10.1111/ace.13581>
- Roig-Soriano, J., Sánchez-de-Diego, C., Esandi-Jauregui, J., Verdés, S., Abraham, C. R., Bosch, A., Ventura, F., & Chillón, M. (2023). Differential toxicity profile of secreted and processed α -Klotho expression over mineral metabolism and bone microstructure. *Scientific Reports*, 13(1). <https://doi.org/10.1038/s41598-023-31117-6>
- Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., ... Brown, R. H. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, 362(6415), 59–62. <https://doi.org/10.1038/362059a0>
- Rothstein, J. D., Jin, L., Dykes-Hoberg, M., & Kuncl, R. W. (1993). Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. *Proceedings of the National Academy of Sciences*, 90(14), 6591–6595. <https://doi.org/10.1073/pnas.90.14.6591>
- Rueggsegger, C., Maharjan, N., Goswami, A., Filézac de L'Etang, A., Weis, J., Troost, D., Heller, M., Gut, H., & Saxena, S. (2016). Aberrant association of misfolded SOD1 with Na⁺/K⁺-ATPase- α 3 impairs its activity and contributes to motor neuron vulnerability in ALS. *Acta Neuropathologica*, 131(3), 427–451. <https://doi.org/10.1007/s00401-015-1510-4>
- Ruhl, D. A., Bomba-Warczak, E., Watson, E. T., Bradberry, M. M., Peterson, T. A., Basu, T., Frelka, A., Evans, C. S., Briguglio, J. S., Basta, T., Stowell, M. H. B., Savas, J. N., Roopra, A., Pearce, R. A., Piper, R. C., & Chapman, E. R. (2019). Synaptotagmin 17 controls neurite outgrowth and synaptic physiology via distinct cellular pathways. *Nature Communications*, 10(1), 3532. <https://doi.org/10.1038/s41467-019-11459-4>
- Ryan, S., Rollinson, S., Hobbs, E., & Pickering-Brown, S. (2022). C9orf72 dipeptides disrupt the nucleocytoplasmic transport machinery and cause TDP-43 mislocalisation to the cytoplasm. *Scientific Reports*, 12(1), 4799. <https://doi.org/10.1038/s41598-022-08724-w>
- Saba, L., Viscomi, M. T., Caioli, S., Pignataro, A., Bisicchia, E., Pieri, M., Molinari, M., Ammassari-Teule, M., & Zona, C. (2016). Altered Functionality, Morphology, and Vesicular Glutamate Transporter Expression of Cortical Motor Neurons from a Presymptomatic Mouse Model of Amyotrophic Lateral Sclerosis. *Cerebral Cortex*, 26(4), 1512–1528. <https://doi.org/10.1093/cercor/bhu317>
- Sahu, A., Clemens, Z. J., Shinde, S. N., Sivakumar, S., Pius, A., Bhatia, A., Picciolini, S., Carlomagno, C., Gualerzi, A., Bedoni, M., Van Houten, B., Lovalekar, M., Fitz, N. F., Lefterov, I., Barchowsky, A., Koldamova, R., & Ambrosio, F. (2021). Regulation of aged skeletal muscle regeneration by circulating extracellular vesicles. *Nature Aging*, 1(12), 1148–1161. <https://doi.org/10.1038/s43587-021-00143-2>
- Sahu, A., Mamiya, H., Shinde, S. N., Cheikhi, A., Winter, L. L., Vo, N. V., Stolz, D., Roginskaya, V., Tang, W. Y., St. Croix, C., Sanders, L. H., Franti, M., Van Houten, B., Rando, T. A., Barchowsky, A., & Ambrosio, F. (2018). Age-related declines in α -Klotho drive progenitor cell mitochondrial dysfunction and impaired muscle regeneration. *Nature Communications*, 9(1). <https://doi.org/10.1038/s41467-018-07253-3>
- Samulski, R. J., Berns, K. I., Tan, M., & Muzyczka, N. (1982). Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proceedings of the National Academy of Sciences*, 79(6), 2077–2081. <https://doi.org/10.1073/pnas.79.6.2077>
- Sasaguri, H., Chew, J., Xu, Y.-F., Gendron, T. F., Garrett, A., Lee, C. W., Jansen-West, K., Bauer, P. O., Perkerson, E. A., Tong, J., Stettler, C., & Zhang, Y.-J. (2016). The extreme N-terminus of TDP-43 mediates the cytoplasmic aggregation of TDP-43 and associated toxicity in vivo. *Brain Research*, 1647, 57–64. <https://doi.org/10.1016/j.brainres.2016.04.069>

- Saxena, S., Cabuy, E., & Caroni, P. (2009). A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. *Nature Neuroscience*, 12(5), 627–636. <https://doi.org/10.1038/nn.2297>
- Saxena, S., Roselli, F., Singh, K., Leptien, K., Julien, J.-P., Gros-Louis, F., & Caroni, P. (2013). Neuroprotection through Excitability and mTOR Required in ALS Motoneurons to Delay Disease and Extend Survival. *Neuron*, 80(1), 80–96. <https://doi.org/10.1016/j.neuron.2013.07.027>
- Scaricamazza, S., Salvatori, I., Giacobazzo, G., Loeffler, J. P., Renè, F., Rosina, M., Quessada, C., Proietti, D., Heil, C., Rossi, S., Battistini, S., Giannini, F., Volpi, N., Steyn, F. J., Ngo, S. T., Ferraro, E., Madaro, L., Coccurello, R., Valle, C., & Ferri, A. (2020). Skeletal-Muscle Metabolic Reprogramming in ALS-SOD1G93A Mice Predates Disease Onset and Is A Promising Therapeutic Target. *iScience*, 23(5), 101087. <https://doi.org/10.1016/j.isci.2020.101087>
- Schmidt, M. F., Gan, Z. Y., Komander, D., & Dewson, G. (2021). Ubiquitin signalling in neurodegeneration: mechanisms and therapeutic opportunities. *Cell Death & Differentiation*, 28(2), 570–590. <https://doi.org/10.1038/s41418-020-00706-7>
- Schmidt, M. L., Carden, M. J., Lee, V. M., & Trojanowski, J. Q. (1987). Phosphate dependent and independent neurofilament epitopes in the axonal swellings of patients with motor neuron disease and controls. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, 56(3), 282–294.
- Schutz, B. (2005). Imbalanced excitatory to inhibitory synaptic input precedes motor neuron degeneration in an animal model of amyotrophic lateral sclerosis. *Neurobiology of Disease*, 20(1), 131–140. <https://doi.org/10.1016/j.nbd.2005.02.006>
- Sekiyama, N., Takaba, K., Maki-Yonekura, S., Akagi, K., Ohtani, Y., Imamura, K., Terakawa, T., Yamashita, K., Inaoka, D., Yonekura, K., Kodama, T. S., & Tochio, H. (2022). ALS mutations in the TIA-1 prion-like domain trigger highly condensed pathogenic structures. *Proceedings of the National Academy of Sciences*, 119(38). <https://doi.org/10.1073/pnas.2122523119>
- Semba, R. D., Cappola, A. R., Sun, K., Bandinelli, S., Dalal, M., Crasto, C., Guralnik, J. M., & Ferrucci, L. (2011). Plasma Klotho and Cardiovascular Disease in Adults. *Journal of the American Geriatrics Society*, 59(9), 1596–1601. <https://doi.org/10.1111/j.1532-5415.2011.03558.x>
- Semba, R. D., Moghekar, A. R., Hu, J., Sun, K., Turner, R., Ferrucci, L., & O'Brien, R. (2014). Klotho in the cerebrospinal fluid of adults with and without Alzheimer's disease. *Neuroscience Letters*, 558, 37–40. <https://doi.org/10.1016/j.neulet.2013.10.058>
- Shaker, M. R., Aguado, J., Chaggar, H. K., & Wolvetang, E. J. (2021). Klotho inhibits neuronal senescence in human brain organoids. *Npj Aging and Mechanisms of Disease*, 7(1), 18. <https://doi.org/10.1038/s41514-021-00070-x>
- Shaw, P. J. (2005). Molecular and cellular pathways of neurodegeneration in motor neurone disease. In *Journal of Neurology, Neurosurgery and Psychiatry* (Vol. 76, Issue 8, pp. 1046–1057). BMJ Publishing Group. <https://doi.org/10.1136/jnnp.2004.048652>
- Shayya, L., Babu, S., Piro, E. P., Li, J., & Li, Y. (2018). Distal Predominance of Electrodiagnostic Abnormalities in Early-Stage Amyotrophic Lateral Sclerosis. *Muscle & Nerve*, 58(3), 389–395. <https://doi.org/10.1002/mus.26158>
- Shefner, J. M., Cudkowicz, M., & Brown, R. H. (2006). Motor unit number estimation predicts disease onset and survival in a transgenic mouse model of amyotrophic lateral sclerosis. *Muscle & Nerve*, 34(5), 603–607. <https://doi.org/10.1002/mus.20628>
- Shi, Y., Lin, S., Staats, K. A., Li, Y., Chang, W. H., Hung, S. T., Hendricks, E., Linares, G. R., Wang, Y., Son, E. Y., Wen, X., Kislser, K., Wilkinson, B., Menendez, L., Sugawara, T., Woolwine, P., Huang, M., Cowan, M. J., Ge, B., ... Ichida, J. K. (2018). Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. *Nature Medicine*, 24(3), 313–325. <https://doi.org/10.1038/nm.4490>
- Shibata, N. (1994). Cu Zn superoxide dismutase-like immunoreactivity in Lewy body-like inclusions of sporadic amyotrophic lateral sclerosis. *Neuroscience Letters*, 179(1–2), 149.
- Shiozaki, M., Yoshimura, K., Shibata, M., Koike, M., Matsuura, N., Uchiyama, Y., & Gotow, T. (2008). Morphological and biochemical signs of age-related neurodegenerative changes in klotho mutant mice. *Neuroscience*, 152(4), 924–941. <https://doi.org/10.1016/j.neuroscience.2008.01.032>
- Shiraki-Iida, T., Aizawa, H., Matsumura, Y., Sekine, S., Iida, A., Anazawa, H., Nagai, R., Kuro-o, M., & Nabeshima, Y. (1998). Structure of the mouse klotho gene and its two transcripts encoding membrane and secreted protein. *FEBS Letters*, 424(1–2), 6–10. [https://doi.org/10.1016/S0014-5793\(98\)00127-6](https://doi.org/10.1016/S0014-5793(98)00127-6)

- Si, Y., Cui, X., Kim, S., Wians, R., Sorge, R., Oh, S. J., Kwan, T., AlSharabati, M., Lu, L., Claussen, G., Anderson, T., Yu, S., Morgan, D., Kazamel, M., & King, P. H. (2014). Smads as muscle biomarkers in amyotrophic lateral sclerosis. *Annals of Clinical and Translational Neurology*, 1(10), 778–787. <https://doi.org/10.1002/acn3.117>
- Skopenkova, V. V., Egorova, T. V., & Bardina, M. V. (2021). Muscle-Specific Promoters for Gene Therapy. *Acta Naturae*, 13(1), 47–58. <https://doi.org/10.32607/actanaturae.11063>
- Smith, R. A., Miller, T. M., Yamanaka, K., Monia, B. P., Condon, T. P., Hung, G., Lobsiger, C. S., Ward, C. M., McAlonis-Downes, M., Wei, H., Wancewicz, E. V., Bennett, C. F., & Cleveland, D. W. (2006). Antisense oligonucleotide therapy for neurodegenerative disease. *Journal of Clinical Investigation*, 116(8), 2290–2296. <https://doi.org/10.1172/JCI25424>
- Smith, R. C., O'Bryan, L. M., Farrow, E. G., Summers, L. J., Clinkenbeard, E. L., Roberts, J. L., Cass, T. A., Saha, J., Broderick, C., Ma, Y. L., Zeng, Q. Q., Kharitonov, A., Wilson, J. M., Guo, Q., Sun, H., Allen, M. R., Burr, D. B., Breyer, M. D., & White, K. E. (2012). Circulating αKlotho influences phosphate handling by controlling FGF23 production. *Journal of Clinical Investigation*, 122(12), 4710–4715. <https://doi.org/10.1172/JCI64986>
- Son, Y.-J., & Thompson, W. J. (1995). Nerve sprouting in muscle is induced and guided by processes extended by schwann cells. *Neuron*, 14(1), 133–141. [https://doi.org/10.1016/0896-6273\(95\)90247-3](https://doi.org/10.1016/0896-6273(95)90247-3)
- Sonntag, F., Bleker, S., Leuchs, B., Fischer, R., & Kleinschmidt, J. A. (2006). Adeno-Associated Virus Type 2 Capsids with Externalized VP1/VP2 Trafficking Domains Are Generated prior to Passage through the Cytoplasm and Are Maintained until Uncoating Occurs in the Nucleus. *Journal of Virology*, 80(22), 11040–11054. <https://doi.org/10.1128/JVI.01056-06>
- Spreux-Varoquaux, O., Bensimon, G., Lacomblez, L., Salachas, F., Pradat, P. F., Le Forestier, N., Marouan, A., Dib, M., & Meininger, V. (2002). Glutamate levels in cerebrospinal fluid in amyotrophic lateral sclerosis: a reappraisal using a new HPLC method with coulometric detection in a large cohort of patients. *Journal of the Neurological Sciences*, 193(2), 73–78. [https://doi.org/10.1016/S0022-510X\(01\)00661-X](https://doi.org/10.1016/S0022-510X(01)00661-X)
- Sreedharan, J., Blair, I. P., Tripathi, V. B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J. C., Williams, K. L., Buratti, E., Baralle, F., de Belleruche, J., Mitchell, J. D., Leigh, P. N., Al-Chalabi, A., Miller, C. C., Nicholson, G., & Shaw, C. E. (2008). TDP-43 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. *Science*, 319(5870), 1668–1672. <https://doi.org/10.1126/science.1154584>
- Stagg, S. M., Yoshioka, C., Davulcu, O., & Chapman, M. S. (2022). Cryo-electron Microscopy of Adeno-associated Virus. *Chemical Reviews*, 122(17), 14018–14054. <https://doi.org/10.1021/acs.chemrev.1c00936>
- Stallings, N. R., Puttaparthi, K., Luther, C. M., Burns, D. K., & Elliott, J. L. (2010). Progressive motor weakness in transgenic mice expressing human TDP-43. *Neurobiology of Disease*, 40(2), 404–414. <https://doi.org/10.1016/j.nbd.2010.06.017>
- Stoica, L., Todeasa, S. H., Cabrera, G. T., Salameh, J. S., Elmallah, M. K., Mueller, C., Brown, R. H., & Sena-Esteves, M. (2016). Adeno-associated virus-delivered artificial microRNA extends survival and delays paralysis in an amyotrophic lateral sclerosis mouse model. *Annals of Neurology*, 79(4), 687–700. <https://doi.org/10.1002/ana.24618>
- Sugiura, H., Yoshida, T., Mitobe, M., Yoshida, S., Shiohira, S., Nitta, K., & Tsuchiya, K. (2010). Klotho reduces apoptosis in experimental ischaemic acute kidney injury via HSP-70. *Nephrology Dialysis Transplantation*, 25(1), 60–68. <https://doi.org/10.1093/ndt/gfp451>
- Sui, X., Yoshioka, H., Fukumoto, Y., Kanemaru, K., & Kinouchi, H. (2021). Neuroprotective roles of HAX-1 in ischemic neuronal injury. *Experimental Neurology*, 339, 113642. <https://doi.org/10.1016/j.expneurol.2021.113642>
- Sumiyoshi, H., Mor, N., Lee, S. Y., Doty, S., Henderson, S., Tanaka, S., Yoshioka, H., Rattan, S., & Ramirez, F. (2004). Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. *The Journal of Cell Biology*, 166(4), 591–600. <https://doi.org/10.1083/jcb.200402054>
- Sunico, C. R., Domínguez, G., García-Verdugo, J. M., Osta, R., Montero, F., & Moreno-López, B. (2011). Reduction in the Motoneuron Inhibitory/Excitatory Synaptic Ratio in an Early-Symptomatic Mouse Model of Amyotrophic Lateral Sclerosis. *Brain Pathology*, 21(1), 1–15. <https://doi.org/10.1111/j.1750-3639.2010.00417.x>
- Suzuki, M., McHugh, J., Tork, C., Shelley, B., Hayes, A., Bellantuono, I., Aebischer, P., & Svendsen, C. N. (2008). Direct muscle delivery of GDNF with human mesenchymal stem cells improves motor neuron survival and

- function in a rat model of familial ALS. *Molecular Therapy*, 16(12), 2002–2010. <https://doi.org/10.1038/mt.2008.197>
- Szebényi, K., Wenger, L. M. D., Sun, Y., Dunn, A. W. E., Limegrover, C. A., Gibbons, G. M., Conci, E., Paulsen, O., Mierau, S. B., Balmus, G., & Lakatos, A. (2021). Human ALS/FTD brain organoid slice cultures display distinct early astrocyte and targetable neuronal pathology. *Nature Neuroscience*, 24(11), 1542–1554. <https://doi.org/10.1038/s41593-021-00923-4>
- Tabebordbar, M., Lagerborg, K. A., Stanton, A., King, E. M., Ye, S., Tellez, L., Krunnusz, A., Tavakoli, S., Widrick, J. J., Messemer, K. A., Troiano, E. C., Moghadaszadeh, B., Peacker, B. L., Leacock, K. A., Horwitz, N., Beggs, A. H., Wagers, A. J., & Sabeti, P. C. (2021). Directed evolution of a family of AAV capsid variants enabling potent muscle-directed gene delivery across species. *Cell*, 184(19), 4919–4938.e22. <https://doi.org/10.1016/j.cell.2021.08.028>
- Tadic, V., Prell, T., Lautenschlaeger, J., & Grosskreutz, J. (2014). The ER mitochondria calcium cycle and ER stress response as therapeutic targets in amyotrophic lateral sclerosis. *Frontiers in Cellular Neuroscience*, 8. <https://doi.org/10.3389/fncel.2014.00147>
- Takeuchi, T., Maeta, K., Ding, X., Oe, Y., Takeda, A., Inoue, M., Nagano, S., Fujihara, T., Matsuda, S., Ishigaki, S., Sahashi, K., Minakawa, E. N., Mochizuki, H., Neya, M., Sobue, G., & Nagai, Y. (2023). Sustained therapeutic benefits by transient reduction of TDP-43 using ENA-modified antisense oligonucleotides in ALS/FTD mice. *Molecular Therapy - Nucleic Acids*, 31, 353–366. <https://doi.org/10.1016/j.omtn.2023.01.006>
- Tateno, M., Kato, S., Sakurai, T., Nukina, N., Takahashi, R., & Araki, T. (2009). Mutant SOD1 impairs axonal transport of choline acetyltransferase and acetylcholine release by sequestering KAP3. *Human Molecular Genetics*, 18(5), 942–955. <https://doi.org/10.1093/hmg/ddn422>
- Tateno, M., Sadakata, H., Tanaka, M., Itohara, S., Shin, R.-M., Miura, M., Masuda, M., Aosaki, T., Urushitani, M., Misawa, H., & Takahashi, R. (2004). Calcium-permeable AMPA receptors promote misfolding of mutant SOD1 protein and development of amyotrophic lateral sclerosis in a transgenic mouse model. *Human Molecular Genetics*, 13(19), 2183–2196. <https://doi.org/10.1093/hmg/ddh246>
- Tawfik, B., Martins, J. S., Houy, S., Imig, C., Pinheiro, P. S., Wojcik, S. M., Brose, N., Cooper, B. H., & Sørensen, J. B. (2021). Synaptotagmin-7 places dense-core vesicles at the cell membrane to promote Munc13-2- and Ca²⁺-dependent priming. *ELife*, 10. <https://doi.org/10.7554/eLife.64527>
- Taylor, J. P., Brown, R. H., & Cleveland, D. W. (2016). Decoding ALS: From genes to mechanism. In *Nature* (Vol. 539, Issue 7628, pp. 197–206). Nature Publishing Group. <https://doi.org/10.1038/nature20413>
- Thams, S., Brodin, P., Plantman, S., Saxelin, R., Kärre, K., & Cullheim, S. (2009). Classical Major Histocompatibility Complex Class I Molecules in Motoneurons: New Actors at the Neuromuscular Junction. *The Journal of Neuroscience*, 29(43), 13503–13515. <https://doi.org/10.1523/JNEUROSCI.0981-09.2009>
- Thomsen, G. M., Alkaslasi, M., Vit, J. P., Lawless, G., Godoy, M., Gowing, G., Shelest, O., & Svendsen, C. N. (2017). Systemic injection of AAV9-GDNF provides modest functional improvements in the SOD1 G93A ALS rat but has adverse side effects. *Gene Therapy*, 24(4), 245–252. <https://doi.org/10.1038/gt.2017.9>
- Thomsen, G. M., Gowing, G., Latter, J., Chen, M., Vit, J. P., Staggenborg, K., Avalos, P., Alkaslasi, M., Ferraiuolo, L., Likhite, S., Kaspar, B. K., & Svendsen, C. N. (2014). Delayed disease onset and extended survival in the SOD1G93A rat model of amyotrophic lateral sclerosis after suppression of mutant SOD1 in the motor cortex. *Journal of Neuroscience*, 34(47), 15587–15600. <https://doi.org/10.1523/JNEUROSCI.2037-14.2014>
- Todd, T. W., & Petrucelli, L. (2022). Modelling amyotrophic lateral sclerosis in rodents. *Nature Reviews Neuroscience*, 23(4), 231–251. <https://doi.org/10.1038/s41583-022-00564-x>
- Tohgi, H. (1999). Remarkable increase in cerebrospinal fluid 3-nitrotyrosine in patients with sporadic amyotrophic lateral sclerosis. *Annals of Neurology*, 46(1), 129.
- Tohyama, O., Imura, A., Iwano, A., Freund, J.-N., Henrissat, B., Fujimori, T., & Nabeshima, Y. (2004). Klotho Is a Novel β -Glucuronidase Capable of Hydrolyzing Steroid β -Glucuronides. *Journal of Biological Chemistry*, 279(11), 9777–9784. <https://doi.org/10.1074/jbc.M312392200>
- Tolosa, L., Caraballo-Miralles, V., Olmos, G., & Lladó, J. (2011). TNF- α potentiates glutamate-induced spinal cord motoneuron death via NF- κ B. *Molecular and Cellular Neuroscience*, 46(1), 176–186. <https://doi.org/10.1016/j.mcn.2010.09.001>
- Torres, P., Cacabelos, D., Pairada, J., Bauer, K. C., Boada, J., Fontdevila, L., Rossi, C., Povedano, M., Ferrer, I., Pamplona, R., Finlay, B. B., Portero-Otin, M., & Ayala, V. (2020). Gender-Specific Beneficial Effects of

- Docosahexaenoic Acid Dietary Supplementation in G93A-SOD1 Amyotrophic Lateral Sclerosis Mice. *Neurotherapeutics*, 17(1), 269–281. <https://doi.org/10.1007/s13311-019-00808-2>
- Towne, C., Raoul, C., Schneider, B. L., & Aebischer, P. (2008). Systemic AAV6 Delivery Mediating RNA Interference Against SOD1: Neuromuscular Transduction Does Not Alter Disease Progression in fALS Mice. *Molecular Therapy*, 16(6), 1018–1025. <https://doi.org/10.1038/mt.2008.73>
- Towne, C., Setola, V., Schneider, B. L., & Aebischer, P. (2011). Neuroprotection by gene therapy targeting mutant SOD1 in individual pools of motor neurons does not translate into therapeutic benefit in fALS mice. *Molecular Therapy*, 19(2), 274–283. <https://doi.org/10.1038/mt.2010.260>
- Trist, B. G., Fifita, J. A., Hogan, A., Grima, N., Smith, B., Troakes, C., Vance, C., Shaw, C., Al-Sarraj, S., Blair, I. P., & Double, K. L. (2022). Co-deposition of SOD1, TDP-43 and p62 proteinopathies in ALS: evidence for multifaceted pathways underlying neurodegeneration. *Acta Neuropathologica Communications*, 10(1), 122. <https://doi.org/10.1186/s40478-022-01421-9>
- Trolese, M. C., Scarpa, C., Melfi, V., Fabbriozio, P., Sironi, F., Rossi, M., Bendotti, C., & Nardo, G. (2022). Boosting the peripheral immune response in the skeletal muscles improved motor function in ALS transgenic mice. *Molecular Therapy*, 30(8), 2760–2784. <https://doi.org/10.1016/j.ymthe.2022.04.018>
- Trotti, D., Rolfs, A., Danbolt, N. C., Brown, R. H., & Hediger, M. A. (1999). SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. *Nature Neuroscience*, 2(5), 427–433. <https://doi.org/10.1038/8091>
- Tsitkanou, S., Gatta, P. A. D., & Russell, A. P. (2016). Skeletal muscle satellite cells, mitochondria, and MicroRNAs: Their involvement in the pathogenesis of ALS. *Frontiers in Physiology*, 7(SEP), 1–9. <https://doi.org/10.3389/fphys.2016.00403>
- Tsujikawa, H., Kurotaki, Y., Fujimori, T., Fukuda, K., & Nabeshima, Y.-I. (2003). Klotho, a Gene Related to a Syndrome Resembling Human Premature Aging, Functions in a Negative Regulatory Circuit of Vitamin D Endocrine System. *Molecular Endocrinology*, 17(12), 2393–2403. <https://doi.org/10.1210/me.2003-0048>
- Tu, P. H., Raju, P., Robinson, K. A., Gurney, M. E., Trojanowski, J. Q., & Lee, V. M. Y. (1996). Transgenic mice carrying a human mutant superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. *Proceedings of the National Academy of Sciences of the United States of America*, 93(7), 3155–3160. <https://doi.org/10.1073/pnas.93.7.3155>
- Tung, Y. T., Peng, K. C., Chen, Y. C., Yen, Y. P., Chang, M., Thams, S., & Chen, J. A. (2019). Mir-17~92 Confers Motor Neuron Subtype Differential Resistance to ALS-Associated Degeneration. *Cell Stem Cell*, 25(2), 193–209.e7. <https://doi.org/10.1016/j.stem.2019.04.016>
- Turner, B. J., Ackerley, S., Davies, K. E., & Talbot, K. (2010). Dismutase-competent SOD1 mutant accumulation in myelinating Schwann cells is not detrimental to normal or transgenic ALS model mice. *Human Molecular Genetics*, 19(5), 815–824. <https://doi.org/10.1093/hmg/ddp550>
- Turner, B. J., & Talbot, K. (2008). Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. In *Progress in Neurobiology* (Vol. 85, Issue 1, pp. 94–134). <https://doi.org/10.1016/j.pneurobio.2008.01.001>
- Uchida, A., Komiya, Y., Tashiro, T., Yorifuji, H., Kishimoto, T., Nabeshima, Y., & Hisanaga, S. I. (2001). Neurofilaments of Klotho, the mutant mouse prematurely displaying symptoms resembling human aging. *Journal of Neuroscience Research*, 64(4), 364–370. <https://doi.org/10.1002/jnr.1087>
- Urushitani, M., Nakamizo, T., Inoue, R., Sawada, H., Kihara, T., Honda, K., Akaike, A., & Shimohama, S. (2001). N-methyl-D-aspartate receptor-mediated mitochondrial Ca(2+) overload in acute excitotoxic motor neuron death: a mechanism distinct from chronic neurotoxicity after Ca(2+) influx. *Journal of Neuroscience Research*, 63(5), 377–387. [https://doi.org/10.1002/1097-4547\(20010301\)63:5<377::AID-JNR1032>3.0.CO;2-#](https://doi.org/10.1002/1097-4547(20010301)63:5<377::AID-JNR1032>3.0.CO;2-#)
- Utsugi, T., Ohno, T., Ohyama, Y., Uchiyama, T., Saito, Y., Matsumura, Y., Aizawa, H., Itoh, H., Kurabayashi, M., Kawazu, S., Tomono, S., Oka, Y., Suga, T., Kuro-o, M., Nabeshima, Y., & Nagai, R. (2000). Decreased insulin production and increased insulin sensitivity in the klotho mutant mouse, a novel animal model for human aging. *Metabolism*, 49(9), 1118–1123. <https://doi.org/10.1053/meta.2000.8606>
- Vahsen, B. F., Nalluru, S., Morgan, G. R., Farrimond, L., Carroll, E., Xu, Y., Cramb, K. M. L., Amein, B., Scaber, J., Katsikoudi, A., Candalija, A., Carcolé, M., Dafinca, R., Isaacs, A. M., Wade-Martins, R., Gray, E., Turner, M. R., Cowley, S. A., & Talbot, K. (2023). C9orf72-ALS human iPSC microglia are pro-inflammatory and toxic to co-cultured motor neurons via MMP9. *Nature Communications*, 14(1), 5898. <https://doi.org/10.1038/s41467-023-41603-0>

- Valdez, G., Heyer, M. P., Feng, G., & Sanes, J. R. (2014). The role of muscle microRNAs in repairing the neuromuscular junction. *PLoS One*, 9(3), e93140. <https://doi.org/10.1371/journal.pone.0093140>
- Valori, C. F., Brambilla, L., Martorana, F., & Rossi, D. (2014). The multifaceted role of glial cells in amyotrophic lateral sclerosis. *Cellular and Molecular Life Sciences*, 71(2), 287–297. <https://doi.org/10.1007/s00018-013-1429-7>
- Van Alstyne, M., Tattoli, I., Delestrée, N., Recinos, Y., Workman, E., Shihabuddin, L. S., Zhang, C., Mentis, G. Z., & Pellizzoni, L. (2021). Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit. *Nature Neuroscience*, 24(7), 930–940. <https://doi.org/10.1038/s41593-021-00827-3>
- van den Bos, M. A. J., Geevasinga, N., Higashihara, M., Menon, P., & Vucic, S. (2019). Pathophysiology and Diagnosis of ALS: Insights from Advances in Neurophysiological Techniques. *International Journal of Molecular Sciences*, 20(11), 2818. <https://doi.org/10.3390/ijms20112818>
- Van Den Bosch, L., Van Damme, P., Bogaert, E., & Robberecht, W. (2006). The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. *Biochimica et Biophysica Acta*, 1762(11–12), 1068–1082. <https://doi.org/10.1016/j.bbadis.2006.05.002>
- van den Hoff, M. J. B., Labruyère, W. T., Moorman, A. F. M., & Lamers, W. H. (1993). Mammalian gene expression is improved by use of a longer SV₄₀ early polyadenylation cassette. *Nucleic Acids Research*, 21(21), 4987–4988. <https://doi.org/10.1093/nar/21.21.4987>
- Van Hoecke, A., Schoonaert, L., Lemmens, R., Timmers, M., Staats, K. A., Laird, A. S., Peeters, E., Philips, T., Goris, A., Dubois, B., Andersen, P. M., Al-Chalabi, A., Thijs, V., Turnley, A. M., van Vught, P. W., Veldink, J. H., Hardiman, O., Van Den Bosch, L., Gonzalez-Perez, P., ... Robberecht, W. (2012). EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. *Nature Medicine*, 18(9), 1418–1422. <https://doi.org/10.1038/nm.2901>
- Vance, C., Rogelj, B., Hortobágyi, T., De Vos, K. J., Nishimura, A. L., Sreedharan, J., Hu, X., Smith, B., Ruddy, D., Wright, P., Ganesalingam, J., Williams, K. L., Tripathi, V., Al-Saraj, S., Al-Chalabi, A., Leigh, P. N., Blair, I. P., Nicholson, G., de Belleruche, J., ... Shaw, C. E. (2009). Mutations in FUS, an RNA Processing Protein, Cause Familial Amyotrophic Lateral Sclerosis Type 6. *Science*, 323(5918), 1208–1211. <https://doi.org/10.1126/science.1165942>
- Vandervore, L. V., Schot, R., Milanese, C., Smits, D. J., Kasteleijn, E., Fry, A. E., Pilz, D. T., Brock, S., Börklü-Yücel, E., Post, M., Bahi-Buisson, N., Sánchez-Soler, M. J., van Slegtenhorst, M., Keren, B., Afenjar, A., Coury, S. A., Tan, W.-H., Oegema, R., de Vries, L. S., ... Mancini, G. M. S. (2019). TMX2 Is a Crucial Regulator of Cellular Redox State, and Its Dysfunction Causes Severe Brain Developmental Abnormalities. *The American Journal of Human Genetics*, 105(6), 1126–1147. <https://doi.org/10.1016/j.ajhg.2019.10.009>
- van Haren, J., Boudeau, J., Schmidt, S., Basu, S., Liu, Z., Lammers, D., Demmers, J., Benhari, J., Grosveld, F., Debant, A., & Galjart, N. (2014). Dynamic Microtubules Catalyze Formation of Navigator-TRIO Complexes to Regulate Neurite Extension. *Current Biology*, 24(15), 1778–1785. <https://doi.org/10.1016/j.cub.2014.06.037>
- Vargas, M. R., Burton, N. C., Gan, L., Johnson, D. A., Schäfer, M., Werner, S., & Johnson, J. A. (2013). Absence of Nrf2 or Its Selective Overexpression in Neurons and Muscle Does Not Affect Survival in ALS-Linked Mutant hSOD1 Mouse Models. *PLoS ONE*, 8(2), e56625. <https://doi.org/10.1371/journal.pone.0056625>
- Vargas, M. R., Johnson, D. A., Sirkis, D. W., Messing, A., & Johnson, J. A. (2008). Nrf2 Activation in Astrocytes Protects against Neurodegeneration in Mouse Models of Familial Amyotrophic Lateral Sclerosis. *The Journal of Neuroscience*, 28(50), 13574–13581. <https://doi.org/10.1523/JNEUROSCI.4099-08.2008>
- Vasu, V. T., Ott, S., Hobson, B., Rashidi, V., Oommen, S., Cross, C. E., & Gohil, K. (2009). Sarcosine and ubiquitin carboxy-terminal hydrolase 1 mRNAs are over-expressed in skeletal muscles of α -tocopherol deficient mice. *Free Radical Research*, 43(2), 106–116. <https://doi.org/10.1080/10715760802616676>
- Veldink, J. H., Bär, P. R., Joosten, E. A. J., Otten, M., Wokke, J. H. J., & van den Berg, L. H. (2003). Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscular Disorders*, 13(9), 737–743. [https://doi.org/10.1016/S0960-8966\(03\)00104-4](https://doi.org/10.1016/S0960-8966(03)00104-4)
- Vielhaber, S., Kunz, D., Winkler, K., Wiedemann, F. R., Kirches, E., Feistner, H., Heinze, H.-J., Elger, C. E., Schubert, W., & Kunz, W. S. (2000). Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis. In *Brain* (Vol. 123).
- Walker, A. K., Spiller, K. J., Ge, G., Zheng, A., Xu, Y., Zhou, M., Tripathy, K., Kwong, L. K., Trojanowski, J. Q., & Lee, V. M.-Y. (2015). Functional recovery in new mouse models of ALS/FTLD after clearance of

- pathological cytoplasmic TDP-43. *Acta Neuropathologica*, 130(5), 643–660. <https://doi.org/10.1007/s00401-015-1460-x>
- Wang, C., Pan, C., Yong, H., Wang, F., Bo, T., Zhao, Y., Ma, B., He, W., & Li, M. (2023). Emerging non-viral vectors for gene delivery. *Journal of Nanobiotechnology*, 21(1), 272. <https://doi.org/10.1186/s12951-023-02044-5>
- Wang, D., Tai, P. W. L., & Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. *Nature Reviews Drug Discovery*, 18(5), 358–378. <https://doi.org/10.1038/s41573-019-0012-9>
- Wang, H., Ghosh, A., Baigude, H., Yang, C., Qiu, L., Xia, X., Zhou, H., Rana, T. M., & Xu, Z. (2008). Therapeutic Gene Silencing Delivered by a Chemically Modified Small Interfering RNA against Mutant SOD1 Slows Amyotrophic Lateral Sclerosis Progression. *Journal of Biological Chemistry*, 283(23), 15845–15852. <https://doi.org/10.1074/jbc.M800834200>
- Wang, H., Yang, B., Qiu, L., Yang, C., Kramer, J., Su, Q., Guo, Y., Brown, R. H., Gao, G., & Xu, Z. (2014). Widespread spinal cord transduction by intrathecal injection of rAAV delivers efficacious RNAi therapy for amyotrophic lateral sclerosis. *Human Molecular Genetics*, 23(3), 668–681. <https://doi.org/10.1093/hmg/ddt454>
- Wang, L., Gutmann, D. H., & Roos, R. P. (2011). Astrocyte loss of mutant SOD1 delays ALS disease onset and progression in G85R transgenic mice. *Human Molecular Genetics*, 20(2), 286–293. <https://doi.org/10.1093/hmg/ddq463>
- Wang, L. J., Lu, Y. Y., Muramatsu, S. ichi, Ikeguchi, K., Fujimoto, K. ichi, Okada, T., Mizukami, H., Matsushita, T., Hanazono, Y., Kume, A., Nagatsu, T., Ozawa, K., & Nakano, I. (2002). Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis. *Journal of Neuroscience*, 22(16), 6920–6928. <https://doi.org/20026668>
- Wang, S., Ichinomiya, T., Savchenko, P., Wang, D., Sawada, A., Li, X., Duong, T., Li, W., Bonds, J. A., Kim, E. J., Miyanochara, A., Roth, D. M., Patel, H. H., Patel, P. M., Tadokoro, T., Marsala, M., & Head, B. P. (2022). Subpial delivery of adeno-associated virus 9-synapsin caveolin-1 (AAV9-SynCav1) preserves motor neuron and neuromuscular junction morphology, motor function, delays disease onset, and extends survival in hSOD1G93A mice. *Theranostics*, 12(12), 5389–5403. <https://doi.org/10.7150/thno.72614>
- Wang, W., Duan, W., Wang, Y., Wen, D., Liu, Y., Li, Z., Hu, H., Cui, H., Cui, C., Lin, H., & Li, C. (2017). Intrathecal Delivery of ssAAV9-DAO Extends Survival in SOD1G93A ALS Mice. *Neurochemical Research*, 42(4), 986–996. <https://doi.org/10.1007/s11064-016-2131-6>
- Wang, W., Wang, L., Lu, J., Siedlak, S. L., Fujioka, H., Liang, J., Jiang, S., Ma, X., Jiang, Z., da Rocha, E. L., Sheng, M., Choi, H., Lerou, P. H., Li, H., & Wang, X. (2016). The inhibition of TDP-43 mitochondrial localization blocks its neuronal toxicity. *Nature Medicine*, 22(8), 869–878. <https://doi.org/10.1038/nm.4130>
- Wang, W., Wen, D., Duan, W., Yin, J., Cui, C., Wang, Y., Li, Z., Liu, Y., & Li, C. (2018). Systemic administration of scAAV9-IGF1 extends survival in SOD1 G93A ALS mice via inhibiting p38 MAPK and the JNK-mediated apoptosis pathway. *Brain Research Bulletin*, 139, 203–210. <https://doi.org/10.1016/j.brainresbull.2018.02.015>
- Wang, Y., Duan, W., Wang, W., Di Wen, Liu, Y., Liu, Y., Li, Z., Hu, H., Lin, H., Cui, C., Li, D., Dong, H., & Li, C. (2016). scAAV9-VEGF prolongs the survival of transgenic ALS mice by promoting activation of M2 microglia and the PI3K/Akt pathway. *Brain Research*, 1648, 1–10. <https://doi.org/10.1016/j.brainres.2016.06.043>
- Warsi, J., Abousaab, A., & Lang, F. (2015). Up-Regulation of Excitatory Amino Acid Transporters EAAT1 and EAAT2 by β -Klotho. *NeuroSignals*, 23(1), 59–70. <https://doi.org/10.1159/000442604>
- Watanabe, S., Oiwa, K., Murata, Y., Komine, O., Sobue, A., Endo, F., Takahashi, E., & Yamanaka, K. (2020). ALS-linked TDP-43M337V knock-in mice exhibit splicing deregulation without neurodegeneration. *Molecular Brain*, 13(1), 8. <https://doi.org/10.1186/s13041-020-0550-4>
- Webster, C. P., Smith, E. F., Bauer, C. S., Moller, A., Hautbergue, G. M., Ferraiuolo, L., Myszczyńska, M. A., Higginbottom, A., Walsh, M. J., Whitworth, A. J., Kaspar, B. K., Meyer, K., Shaw, P. J., Grierson, A. J., & De Vos, K. J. (2016). The C9orf72 protein interacts with Rab1a and the ULK1 complex to regulate initiation of autophagy. *The EMBO Journal*, 35(15), 1656–1676. <https://doi.org/10.15252/embj.201694401>
- Wehling-Henricks, M., Li, Z., Lindsey, C., Wang, Y., Welc, S. S., Ramos, J. N., Khanlou, N., Kuro-o, M., & Tidball, J. G. (2016). Klotho gene silencing promotes pathology in the mdx mouse model of Duchenne muscular dystrophy. *Human Molecular Genetics*, 25(12), ddw111. <https://doi.org/10.1093/hmg/ddw111>

- Welc, S. S., Wehling-Henricks, M., Kuro-o, M., Thomas, K. A., & Tidball, J. G. (2020). Modulation of Klotho expression in injured muscle perturbs Wnt signalling and influences the rate of muscle growth. *Experimental Physiology*, 105(1), 132–147. <https://doi.org/10.1113/EP088142>
- Wen, D., Cui, C., Duan, W., Wang, W., Wang, Y., Liu, Y., Li, Z., & Li, C. (2019). The role of insulin-like growth factor 1 in ALS cell and mouse models: A mitochondrial protector. *Brain Research Bulletin*, 144, 1–13. <https://doi.org/10.1016/j.brainresbull.2018.09.015>
- Whitby, F. G., & Hill, C. P. (2007). A Versatile Platform for Inactivation and Destruction. *Structure*, 15(2), 137–138. <https://doi.org/10.1016/j.str.2007.01.009>
- Wiedemann, F. R., Manfredi, G., Mawrin, C., Flint Beal, M., & Schon, E. A. (2002). Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. In *J. Neurochem* (Vol. 80).
- Wiedemann, F. R., Winkler, K., Kuznetsov, A., Bartels, C., Vielhaber, S., Feistner, H., & Kunz, W. S. (1988). *Impairment of mitochondrial function in skeletal muscle of patients with ALS*.
- Wijesekera, L. C., & Leigh, P. N. (2009). Amyotrophic lateral sclerosis. *Orphanet Journal of Rare Diseases*, 4(1), 1–22. <https://doi.org/10.1186/1750-1172-4-3>
- Williams, A. H., Valdez, G., Moresi, V., Qi, X., McAnally, J., Elliott, J. L., Bassel-Duby, R., Sanes, J. R., & Olson, E. N. (2009). MicroRNA-206 Delays ALS Progression and Promotes Regeneration of Neuromuscular Synapses in Mice. *Science*, 326(5959), 1549–1554. <https://doi.org/10.1126/science.1181046>
- Williams, T. L., Day, N. C., Ince, P. G., Kamboj, R. K., & Shaw, P. J. (1997). Calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors: a molecular determinant of selective vulnerability in amyotrophic lateral sclerosis. *Annals of Neurology*, 42(2), 200–207. <https://doi.org/10.1002/ana.410420211>
- Wils, H., Kleinberger, G., Janssens, J., Pereson, S., Joris, G., Cuijt, I., Smits, V., Ceuterick-de Groote, C., Van Broeckhoven, C., & Kumar-Singh, S. (2010). TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. *Proceedings of the National Academy of Sciences*, 107(8), 3858–3863. <https://doi.org/10.1073/pnas.0912417107>
- Wischhof, L., Scifo, E., Ehninger, D., & Bano, D. (2022). AIFM1 beyond cell death: An overview of this OXPHOS-inducing factor in mitochondrial diseases. *EBioMedicine*, 83, 104231. <https://doi.org/10.1016/j.ebiom.2022.104231>
- Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, 6(5), 359–362. <https://doi.org/10.1038/nmeth.1322>
- Wong, M., & Martin, L. J. (2010). Skeletal muscle-restricted expression of human SOD1 causes motor neuron degeneration in transgenic mice. *Human Molecular Genetics*, 19(11), 2284–2302. <https://doi.org/10.1093/hmg/ddq106>
- Woo, J. S., Jeong, S. Y., Park, J. H., Choi, J. H., & Lee, E. H. (2020). Calsequestrin: a well-known but curious protein in skeletal muscle. *Experimental & Molecular Medicine*, 52(12), 1908–1925. <https://doi.org/10.1038/s12276-020-00535-1>
- Wright, J. D., An, S., Xie, J., Yoon, J., Nischan, N., Kohler, J. J., Oliver, N., Lim, C., & Huang, C. (2017). Modeled structural basis for the recognition of α 2-3-sialyllactose by soluble Klotho. *The FASEB Journal*, 31(8), 3574–3586. <https://doi.org/10.1096/fj.201700043R>
- Wu, D., Lim, E., Vaillant, F., Asselin-Labat, M.-L., Visvader, J. E., & Smyth, G. K. (2010). ROAST: rotation gene set tests for complex microarray experiments. *Bioinformatics*, 26(17), 2176–2182. <https://doi.org/10.1093/bioinformatics/btq401>
- Wu, R., Wang, H., Xia, X., Zhou, H., Liu, C., Castro, M., & Xu, Z. (2009). Nerve injection of viral vectors efficiently transfers transgenes into motor neurons and delivers RNAi therapy against ALS. *Antioxidants and Redox Signaling*, 11(7), 1523–1534. <https://doi.org/10.1089/ars.2009.2618>
- Wu, S.-E., & Chen, W.-L. (2022). Soluble klotho as an effective biomarker to characterize inflammatory states. *Annals of Medicine*, 54(1), 1520–1529. <https://doi.org/10.1080/07853890.2022.2077428>
- Xiao, Z., King, G., Mancarella, S., Munkhsaikhan, U., Cao, L., Cai, C., & Quarles, L. D. (2019). FGF23 expression is stimulated in transgenic α -Klotho longevity mouse model. *JCI Insight*, 4(23). <https://doi.org/10.1172/jci.insight.132820>
- Xie, M., Liu, Y. U., Zhao, S., Zhang, L., Bosco, D. B., Pang, Y.-P., Zhong, J., Sheth, U., Martens, Y. A., Zhao, N., Liu, C.-C., Zhuang, Y., Wang, L., Dickson, D. W., Mattson, M. P., Bu, G., & Wu, L.-J. (2022). TREM2 interacts with TDP-43 and mediates microglial neuroprotection against TDP-43-related neurodegeneration. *Nature Neuroscience*, 25(1), 26–38. <https://doi.org/10.1038/s41593-021-00975-6>

- Xie, N., Zhang, L., Gao, W., Huang, C., Huber, P. E., Zhou, X., Li, C., Shen, G., & Zou, B. (2020). NAD⁺ metabolism: pathophysiologic mechanisms and therapeutic potential. *Signal Transduction and Targeted Therapy*, 5(1), 227. <https://doi.org/10.1038/s41392-020-00311-7>
- Xing, L., Guo, H., Meng, S., Zhu, B., Fang, J., Huang, J., Chen, J., Wang, Y., Wang, L., Yao, X., & Wang, H. (2021). Klotho ameliorates diabetic nephropathy by activating Nrf2 signaling pathway in podocytes. *Biochemical and Biophysical Research Communications*, 534, 450–456. <https://doi.org/10.1016/j.bbrc.2020.11.061>
- Xu, Y., & Sun, Z. (2015). Molecular Basis of Klotho: From Gene to Function in Aging. *Endocrine Reviews*, 36(2), 174–193. <https://doi.org/10.1210/er.2013-1079>
- Xu, Y.-F., Gendron, T. F., Zhang, Y.-J., Lin, W.-L., D'Alton, S., Sheng, H., Casey, M. C., Tong, J., Knight, J., Yu, X., Rademakers, R., Boylan, K., Hutton, M., McGowan, E., Dickson, D. W., Lewis, J., & Petrucelli, L. (2010). Wild-Type Human TDP-43 Expression Causes TDP-43 Phosphorylation, Mitochondrial Aggregation, Motor Deficits, and Early Mortality in Transgenic Mice. *The Journal of Neuroscience*, 30(32), 10851–10859. <https://doi.org/10.1523/JNEUROSCI.1630-10.2010>
- Yamamoto, M., Clark, J. D., Pastor, J. V., Gurnani, P., Nandi, A., Kurosu, H., Miyoshi, M., Ogawa, Y., Castrillon, D. H., Rosenblatt, K. P., & Kuro-O, M. (2005). Regulation of oxidative stress by the anti-aging hormone klotho. *Journal of Biological Chemistry*, 280(45), 38029–38034. <https://doi.org/10.1074/jbc.M509039200>
- Yamanaka, K., Chun, S. J., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D. H., Takahashi, R., Misawa, H., & Cleveland, D. W. (2008). Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nature Neuroscience*, 11(3), 251–253. <https://doi.org/10.1038/nn2047>
- Yamashita, T., Chai, H. L., Teramoto, S., Tsuji, S., Shimazaki, K., Muramatsu, S. ichi, & Kwak, S. (2013a). Rescue of amyotrophic lateral sclerosis phenotype in a mouse model by intravenous AAV9-ADAR2 delivery to motor neurons. *EMBO Molecular Medicine*, 5(11), 1710–1719. <https://doi.org/10.1002/emmm.201302935>
- Yamashita, T., Chai, H. L., Teramoto, S., Tsuji, S., Shimazaki, K., Muramatsu, S. ichi, & Kwak, S. (2013b). Rescue of amyotrophic lateral sclerosis phenotype in a mouse model by intravenous AAV9-ADAR2 delivery to motor neurons. *EMBO Molecular Medicine*, 5(11), 1710–1719. <https://doi.org/10.1002/emmm.201302935>
- Yousef, H., Conboy, M. J., Morgenthaler, A., Schlesinger, C., Bugaj, L., Paliwal, P., Greer, C., Conboy, I. M., & Schaffer, D. (2015). Systemic attenuation of the TGF- β pathway by a single drug simultaneously rejuvenates hippocampal neurogenesis and myogenesis in the same old mammal. *Oncotarget*, 6(14), 11959–11978. <https://doi.org/10.18632/oncotarget.3851>
- Yuan, A., Rao, M. V., Veeranna, & Nixon, R. A. (2012). Neurofilaments at a glance. *Journal of Cell Science*, 125(14), 3257–3263. <https://doi.org/10.1242/jcs.104729>
- Yuan, Q., Ren, Q., Li, L., Tan, H., Lu, M., Tian, Y., Huang, L., Zhao, B., Fu, H., Hou, F. F., Zhou, L., & Liu, Y. (2022). A Klotho-derived peptide protects against kidney fibrosis by targeting TGF- β signaling. *Nature Communications*, 13(1), 438. <https://doi.org/10.1038/s41467-022-28096-z>
- Zeldich, E., Chen, C. Di, Avila, R., Medicetty, S., & Abraham, C. R. (2015). The Anti-Aging Protein Klotho Enhances Remyelination Following Cuprizone-Induced Demyelination. *Journal of Molecular Neuroscience*, 57(2), 185–196. <https://doi.org/10.1007/s12031-015-0598-2>
- Zeldich, E., Chen, C. Di, Boden, E., Howat, B., Nasse, J. S., Zeldich, D., Lambert, A. G., Yuste, A., Cherry, J. D., Mathias, R. M., Ma, Q., Lau, N. C., McKee, A. C., Hatzipetros, T., & Abraham, C. R. (2019). Klotho Is Neuroprotective in the Superoxide Dismutase (SOD1G93A) Mouse Model of ALS. *Journal of Molecular Neuroscience*, 69(2), 264–285. <https://doi.org/10.1007/s12031-019-01356-2>
- Zeldich, E., Chen, C. Di, Colvin, T. A., Bove-Fenderson, E. A., Liang, J., Tucker Zhou, T. B., Harris, D. A., & Abraham, C. R. (2014). The neuroprotective effect of Klotho is mediated via regulation of members of the redox system. *Journal of Biological Chemistry*, 289(35), 24700–24715. <https://doi.org/10.1074/jbc.M114.567321>
- Zeng, C.-Y., Yang, T.-T., Zhou, H.-J., Zhao, Y., Kuang, X., Duan, W., & Du, J.-R. (2019). Lentiviral vector-mediated overexpression of Klotho in the brain improves Alzheimer's disease-like pathology and cognitive deficits in mice. *Neurobiology of Aging*, 78, 18–28. <https://doi.org/10.1016/j.neurobiolaging.2019.02.003>
- Zhang, B., Tu, P., Abtahian, F., Trojanowski, J. Q., & Lee, V. M. (1997). Neurofilaments and orthograde transport are reduced in ventral root axons of transgenic mice that express human SOD1 with a G93A mutation. *The Journal of Cell Biology*, 139(5), 1307–1315. <https://doi.org/10.1083/jcb.139.5.1307>

- Zhang, H., Tan, C.-F., Mori, F., Tanji, K., Kakita, A., Takahashi, H., & Wakabayashi, K. (2008). TDP-43-immunoreactive neuronal and glial inclusions in the neostriatum in amyotrophic lateral sclerosis with and without dementia. *Acta Neuropathologica*, 115(1), 115–122. <https://doi.org/10.1007/s00401-007-0285-7>
- Zhang, K., Daigle, J. G., Cunningham, K. M., Coyne, A. N., Ruan, K., Grima, J. C., Bowen, K. E., Wadhwa, H., Yang, P., Rigo, F., Taylor, J. P., Gitler, A. D., Rothstein, J. D., & Lloyd, T. E. (2018). Stress Granule Assembly Disrupts Nucleocytoplasmic Transport. *Cell*, 173(4), 958–971.e17. <https://doi.org/10.1016/j.cell.2018.03.025>
- Zhang, K., Donnelly, C. J., Haeusler, A. R., Grima, J. C., Machamer, J. B., Steinwald, P., Daley, E. L., Miller, S. J., Cunningham, K. M., Vidensky, S., Gupta, S., Thomas, M. A., Hong, I., Chiu, S.-L., Haganir, R. L., Ostrow, L. W., Matunis, M. J., Wang, J., Sattler, R., ... Rothstein, J. D. (2015). The C9orf72 repeat expansion disrupts nucleocytoplasmic transport. *Nature*, 525(7567), 56–61. <https://doi.org/10.1038/nature14973>
- Zhang, W., Zhang, L., Liang, B., Schroeder, D., Zhang, Z., Cox, G. A., Li, Y., & Lin, D.-T. (2016). Hyperactive somatostatin interneurons contribute to excitotoxicity in neurodegenerative disorders. *Nature Neuroscience*, 19(4), 557–559. <https://doi.org/10.1038/nn.4257>
- Zhang, X., Wang, F., Hu, Y., Chen, R., Meng, D., Guo, L., Lv, H., Guan, J., & Jia, Y. (2020). In vivo stress granule misprocessing evidenced in a FUS knock-in ALS mouse model. *Brain*, 143(5), 1350–1367. <https://doi.org/10.1093/brain/awaa076>
- Zhang, Y.-J., Guo, L., Gonzales, P. K., Gendron, T. F., Wu, Y., Jansen-West, K., O'Raw, A. D., Pickles, S. R., Prudencio, M., Carlomagno, Y., Gachechiladze, M. A., Ludwig, C., Tian, R., Chew, J., DeTure, M., Lin, W.-L., Tong, J., Daugherty, L. M., Yue, M., ... Petrucelli, L. (2019). Heterochromatin anomalies and double-stranded RNA accumulation underlie C9orf72 poly(PR) toxicity. *Science*, 363(6428). <https://doi.org/10.1126/science.aav2606>
- Zhang, Z., Almeida, S., Lu, Y., Nishimura, A. L., Peng, L., Sun, D., Wu, B., Karydas, A. M., Tartaglia, M. C., Fong, J. C., Miller, B. L., Farese, R. V., Moore, M. J., Shaw, C. E., & Gao, F.-B. (2013). Downregulation of MicroRNA-9 in iPSC-Derived Neurons of FTD/ALS Patients with TDP-43 Mutations. *PLoS ONE*, 8(10), e76055. <https://doi.org/10.1371/journal.pone.0076055>
- Zhao, C., Devlin, A., Chouhan, A. K., Selvaraj, B. T., Stavrou, M., Burr, K., Brivio, V., He, X., Mehta, A. R., Story, D., Shaw, C. E., Dando, O., Hardingham, G. E., Miles, G. B., & Chandran, S. (2020). Mutant C9orf72 human iPSC-derived astrocytes cause non-cell autonomous motor neuron pathophysiology. *Glia*, 68(5), 1046–1064. <https://doi.org/10.1002/glia.23761>
- Zhao, M., Murakami, S., Matsumaru, D., Kawauchi, T., Nabeshima, Y., & Motohashi, H. (2022). NRF2 pathway activation attenuates ageing-related renal phenotypes due to α -klotho deficiency. *The Journal of Biochemistry*, 171(5), 579–589. <https://doi.org/10.1093/jb/mvac014>
- Zhao, W., Beers, D. R., Bell, S., Wang, J., Wen, S., Baloh, R. H., & Appel, S. H. (2015). TDP-43 activates microglia through NF- κ B and NLRP3 inflammasome. *Experimental Neurology*, 273, 24–35. <https://doi.org/10.1016/j.expneurol.2015.07.019>
- Zhao, W., Beers, D. R., Hooten, K. G., Sieglaff, D. H., Zhang, A., Kalyana-Sundaram, S., Traini, C. M., Halsey, W. S., Hughes, A. M., Sathe, G. M., Livi, G. P., Fan, G. H., & Appel, S. H. (2017). Characterization of gene expression phenotype in amyotrophic lateral sclerosis monocytes. *JAMA Neurology*, 74(6), 677–685. <https://doi.org/10.1001/jamaneurol.2017.0357>
- Zhou, L., Li, Y., Zhou, D., Tan, R. J., & Liu, Y. (2013). Loss of Klotho Contributes to Kidney Injury by Derepression of Wnt/ β -Catenin Signaling. *Journal of the American Society of Nephrology*, 24(5), 771–785. <https://doi.org/10.1681/ASN.2012080865>
- Zhou, T., Ahmad, T. K., Gozda, K., Truong, J., Kong, J., & Namaka, M. (2017). Implications of white matter damage in amyotrophic lateral sclerosis (review). *Molecular Medicine Reports*, 16(4), 4379–4392. <https://doi.org/10.3892/mmr.2017.7186>
- Zhou, Y., Liu, S., Oztürk, A., & Hicks, G. G. (2014). FUS-regulated RNA metabolism and DNA damage repair: Implications for amyotrophic lateral sclerosis and frontotemporal dementia pathogenesis. *Rare Diseases (Austin, Tex.)*, 2, e29515. <https://doi.org/10.4161/rdis.29515>
- Zhu, L., Stein, L. R., Kim, D., Ho, K., Yu, G.-Q., Zhan, L., Larsson, T. E., & Mucke, L. (2018). Klotho controls the brain-immune system interface in the choroid plexus. *Proceedings of the National Academy of Sciences*, 115(48). <https://doi.org/10.1073/pnas.1808609115>

APPENDIX

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
TROPIC FACTORS								
GDNF								
t.p.: medial and lateral	GM	Ex vivo RV MPC 50,000 /site	PGK1	B6SJL.SOD1 ^{G93A} mouse	P42	0%	Increased large spinal MNs and slowed muscle atrophy. Delayed onset by 15 days and improved motor function.	(Mohajeri et al., 1999)
i.m.: GM + TB	Skeletal muscles	AAV2 (3x10 ¹⁰ + 2x10 ¹⁰ vg)	CMV	SOD1 ^{G93A} male mouse	P63	16.6%	>120-fold GDNF in muscles. Rescued muscle mass loss and atrophied myofibers. Motor function deterioration was delayed.	(Wang et al., 2002)
i.m.: TA + GM + QD + PSM	Skeletal muscles	Ad5 (5x10 ⁹ PFU/muscle)	CMV	B6SJL.SOD1 ^{G93A} mouse	P5-7	12.2%	Delayed onset. Motor performance declined more slowly. MNs preserved for 4 months. No differences in CMAP.	(Acsadi et al., 2002)
i.m.: right GM	GM	Ad5 (1x10 ⁸ PFU)	CMV	B6SJL(SOD1 ^{G93G}) ^{dl} mouse	P245	N/A	No differences in clinical grade, voluntary movement or rotarod. Preserved p-Akt+ large MNs.	(Manabe et al., 2003)
i.m.: QD + InC	Spinal MNs	AAV2 (1x10 ¹⁰ vp)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60, 90	8.9%	Delayed onset (17.5%). Did not slow disease progression.	(Kaspar et al., 2003)
i.s.; i.c.: FMN	Lumbar or facial MNs	LV (50/100 ng p24 antigen/site)	PGK1	B6SJL.SOD1 ^{G93A} mouse	P40	N/A	Appearance of SFP in GM was not delayed and GM CMAP was not preserved. Local GDNF did not prevent lumbar MNs loss.	(Guillot et al., 2004)
t.p.: lumbar spinal cord	Spinal cord	Ex vivo LV NPC (1.2x10 ⁵ bilateral)	PGK1	SD SOD1 ^{G93A} rats	P90	0%	GDNF correlated with increased ChAT expression within axons. MNs not preserved. No differences in BBB scores.	(Klein, et al., 2005)
i.m.: TA + TB + LT	Spinal MNs	Ex vivo LV MSC; 3 times (120,000 /site)	PGK1	SD SOD1 ^{G93A} rats	P80	18d	Decreased denervation of NMJs, maintained MNs and delayed onset by 28 days. Motor decline (BBB test) at a slower rate.	(Suzuki et al., 2008)
i.v.: tail	Broad	AAV9 (4x10 ¹² vp)	CAG	SD SOD1 ^{G93A} rats	P25	0%	Modestly improved grip strength and delayed forelimb paralysis. Slower weight gain, reduced activity and working memory.	(Thomsen et al., 2017)
i.v. tail	Skeletal muscles	AAV8 (1.8x10 ¹⁴ vg/kg)	Desmin	C57BL6.SOD1 ^{G93A} mouse	P35	N/A	Reduced glial reactivity and preserved LMNs and MNJ. Delayed onset and slowed ALS progression.	(Módol-Caballero, et al., 2021a)
IGF-1								
i.m.: QD + InC	Spinal MNs	AAV2 (1x10 ¹⁰ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60; P90	30%; 17.7%	Delayed onset (34%) and motor deficits. 34% more surviving MNs at end stage. Reduced apoptosis, astrogliosis and aggregates.	(Kaspar et al., 2003)
i.s.: L4 and L5 bilateral	Spinal MNs	AAV2 (1.2x10 ¹⁰ vg/site)	CAG	B6SJL.SOD1 ^{G93A} mouse	P60	10.7% (males)	Rescued MNs loss. Delayed onset (12.4%) and slowed grip strength and weight declines, all selectively in males.	(Lepore et al., 2007)
i.c.b.: DCN bilateral	CNS	AAV1, AAV2 (2x10 ¹⁰ vp/site)	CMV	SOD1 ^{G93A} mouse	P90	11.6%	Preserved spinal MNs, in cervical regions only AAV1. Grip strength and rotarod were improved until end stage. Reduced gliosis.	(Dodge et al., 2008)
i.s.: cervical unilateral	Spinal MNs	AAV2 (4.12x10 ¹⁰ vg)	CAG	SD SOD1 ^{G93A} rats	P80	0%	Reduced MNs loss within treated C3-C5 spinal segments. Only males had full preservation of ipsilateral grip strength.	(Franz et al., 2009)
i.m.: GM + TA + InC + TB	MNs or muscles	LV-αCAR or LV-VSV-G (7.1x10 ¹⁰ vg)	CMV	B6.SOD1 ^{G93A} mouse	P28	9% (αCAR)	Disease onset by weight was delayed 27% (αCAR) or 22.7% (VSVG). αCAR preserved MNs and improved rotarod test and gait.	(Eleftheriadou et al., 2016)
i.v.: tail	Broad	scAAV9 (1.5x10 ¹¹ vg)	CMV	B6SJL.SOD1 ^{G93A} female mouse	P90	7.7%	Decreased reactive glia, weight and MN loss. Mitigation of myelin pathology in ventral roots. Inhibition of apoptosis and less TNFα.	(Wang et al., 2018)
i.m.: 8 muscles	MNs	scAAV9 (1x10 ¹⁰ vg/muscle)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60; P90	21.8%; 11.7%	Protection of MNs, locomotion and weight. Less atrophic muscles and reactive glia. Benefits explained by upregulated DAO.	(H. Q. Lin et al., 2018)
i.m.: multiple	MNs	scAAV9 (2-3x10 ¹⁰ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60	N/A	Protection of mitochondria from apoptosis and upregulated mitophagy: increased MMP and decreased CytC release.	(Wen et al., 2019)
IGF-2								

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
i.m.: QD + InC	Spinal MNs	AAV9 (11x10 ¹¹ vg)	CBA	B6.SOD1 ^{G93A} mouse	P80	10%	Improved rotarod test and preserved MNs and axonal density in ventral roots. Induced MNs regeneration and sprouting in NMJ.	(Allodi et al., 2016)
HGF								
i.m.: TA + GM + VL	Spinal MNs	AAV6 (3.12x10 ⁸ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P90	9.8%	No effect on body weight or limb strength. Improved muscle integrity and function could be explained by the FOXO signaling pathway.	(Lee, Lee, et al., 2019)
i.t.: lumbar	Spinal cord	AAV1 (5x10 ⁹ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60	6.5%	No effect on weight. Rotarod, hanging wire and grip strength tests were improved. 2-fold increase in spinal MNs and restored NMJ.	(Lee, Kim, et al., 2019)
VEGF								
i.m.: GM + FCM + DIA + TG + InC	MNs	Rabies-G LV (~9x10 ⁹ TU)	LV (EIAY)	B6SJL.SOD1 ^{G93A} mouse	P21; P90	30%; 14.9%	P21 VEGF delayed onset (28%). Preserved stride length until end stage. MNs survival increased 44-125% depending on the pool.	(Azzouz et al., 2004)
i.c.m.; i.c.: ICap + MC (P50); i.v. (P2, P50)	Spinal and cortical MNs	AAV1, scAAV9 (10 ¹² -10 ¹³ vg/kg)	CMV	LIX1 ^{-/-} cats	P2, 50	N/A	i.c.m resulted in spinal MNs transduction and neurons in the cerebellum, thalamus and MC. i.v. bore low spinal vg and no VEGF protein was detected. No therapeutic effect.	(Bucher et al., 2013)
i.t.: lumbar	Spinal MNs	scAAV9 (1x10 ⁹ vg/g)	CMV	B6SJL.SOD1 ^{G93A} mouse	P90	9.1%	Improved footprint and rotarod test. Preserved MNs and decreased apoptosis and gliosis, switching microglia from a M1 to a M2 state.	(Wang et al., 2016)
NRG1								
i.m.: GM	GM	AAV1 (1x10 ¹¹ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P56; P84	N/A	Nrg1-1 preserved GM CMAP and NMJ by collateral sprouting. Rotarod performance or disease onset did not improve.	(Mancuso et al., 2016)
i.s.: bilateral	Spinal MNs	AAV1 (6 sites; 3.6x10 ⁸ vg)	CAG	B6SJL.SOD1 ^{G93A} mouse	P65	6.5%	Only Nrg1-3, but not Nrg1-1, restored the number of C-buttons and large MNs.	(Lasiene et al., 2016)
i.v.: tail	Skeletal muscles	AAV8 (7.6x10 ¹³ vg/kg)	Desmin	B6SJL.SOD1 ^{G93A} mouse	P42	N/A	Nrg1-1 preserved CMAP of PL, TA and GM with higher MEPs. Preserved NMJ and MNs and decreased gliosis.	(Mòdol-Caballero, et al., 2020b)
i.t.: lumbar	MNs	AAVrh10 (1x10 ¹¹ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P56	N/A	Nrg1-3 preserved NMJ and CMAP of GC, TA and PL. Improved locomotion, surviving MNs and reduced gliosis. Not in males.	(Mòdol-Caballero, et al., 2020a)
i.t. + i.v.: tail	Skeletal muscles, CNS	AAV8; AAVrh10 (10 ¹² vg/kg; 10 ¹¹ vg)	Desmin; CMV	B6SJL.SOD1 ^{G93A} mouse	P42	N/A	Preserved MNs and NMJs, and reduced glial reactivity. Improved motor function and delayed onset. No synergistic effects.	(Mòdol-Caballero, et al., 2021b)
NT-3 + CNTF								
i.m.: unilateral GM + TB + LT	Skeletal muscles	Ad (1x10 ⁹ PFU)	RSV LTR	<i>p_{mn}</i> mouse	P3-5	51.7%	Onset not affected but treated mice had less atrophy and better motor performance. Recovery of GM CMAP and protected axons. Effects were potentiated with AdCNTF.	(Haase et al., 1997, 1998)
CT-1								
i.m.: GM + TB + LT	Skeletal muscles	Ad5 (2.5x10 ⁹ + 1.5x10 ⁹ + 2x10 ⁹ PFU)	RSV LTR	B6SJL.SOD1 ^{G93A} mouse	P5-6	7.7%	Postponed disease onset. Increased GM mass, reduced atrophy and denervation. At end stage, CMAP decreased at a lower rate.	(Bordet et al., 2001)
G-CSF								
i.m.: GM + LT; IS: lumbar	Spinal MNs	AAV1 (IM: 9x10 ⁹ vg; IS: 3.78x10 ⁹ vg)	CBA	B6.SOD1 ^{G93A} female mouse	P70	10%	Contrarily to i.s., i.m. fails to transduce and preserve MNs. i.s. improved strength and rotarod at end stage. Delayed weight loss.	(Henriques et al., 2011)
NDNF								
i.t.	CNS	AAVPHP.eB (5x10 ¹¹ vg)	CBh	C57BL/6.SOD1 ^{G93A} mouse	P61, 85, 100	N/A	Positive effects on locomotion, weight loss, MNs, aggregation and NMJ. Survival mildly extended at P100. No changes in neuroinflammation.	(Cheng et al., 2023)

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
APOPTOSIS								
<i>Bcl-2</i>								
i.s.: bilateral lumbar	Spinal MNs	rAAV (9.5x10 ⁷ vg/site)	CMV	B6SJL.SOD1 ^{G93A} mouse	P35	0%	Onset defined by SFP was delayed (9.8%). At end stage, CMAP amplitude was higher. Rescued ~50% of MNs that degenerate.	(Azzouz, 2000)
<i>SYT13</i>								
i.m.: QD + TM	MNs	AAV9 (11x10 ¹¹ vp)	N/A	B6.SOD1 ^{G93A} mouse	P80	14%	Improved inverted grip test and reduced MNs loss, ER stress and apoptosis. Improved axonal density in ventral roots. Preserved NMJ.	(Nizzardo et al., 2020)
AGGREGATION								
<i>scFv</i>								
i.t.	CNS	scAAV1 (3x10 ⁹ vp)	CMV	B6.SOD1 ^{G37R} mouse	P45	28%	Lifespan correlated with scFv titer. Delayed onset. scFv D3H5 reduced neuronal stress, MNs loss and gliosis by reducing mSOD1.	(Patel et al., 2014)
<i>MIF</i>								
i.s.: lumbar	Spinal cord	AAV9 (5.35x10 ⁸ vg)	CMV	B6.SOD1 ^{G93A} mouse B6.SOD1 ^{G37R} mouse	P1	20.3%	Delayed onset (11.6%), maintained weight, and strength. Reduced mSOD1 but not gliosis. Outcomes enhanced in SOD1 ^{G37R} mice.	(Leyton-Jaimes et al., 2019)
GLUTAMATERGIC								
<i>ADAR2</i>								
i.v.: tail	Upper and lower MNs	AAV9 (2.14x10 ¹² vg)	SYN	ADAR2 ^{flac/lox} /VAcHt-Cre.Fast; AR2 mouse	P63-91 P105	N/A	Model is unable to edit the Q/R site of GluA2 RNA. Treatment ameliorated the decline in rotarod, but not grip strength or weight loss. 10-20% of MNs and TDP-43 mislocalization were rescued.	(Yamashita et al., 2013)
<i>GLT1a</i>								
i.s.: bilateral cervical	Astrocytes	AAV8 (1x10 ¹⁰ vg)	Gfa2	B6.SOD1 ^{G37R} mouse	P110	0%	High expression in astrocytes. PhMNs, NMJ innervation and CMAP of diaphragm were not preserved. Disease progression unaltered.	(Li et al., 2015)
<i>DAO</i>								
i.t.: lumbar	Spinal MNs	AAV9 (1x10 ¹² vg/kg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P90	4.6%	Improved hindlimb stretching, but not motor function. Decreased MNs and muscle atrophy, and NF-κB activation, alleviating gliosis.	(Wang et al., 2017)
MULTITARGET								
<i>IGF-1 + VEGF</i>								
i.c.v.: lateral and 4 th ventricle	Ependymal cells	AAV4 (8x10 ¹⁰ vp)	CMV	B6SJL.SOD1 ^{G93A} mouse	P80-90	12.2%, 6%	VEGF increased survival to a greater extent than IGF-1. Both preserved strength, but not rotarod performance. No additive effect.	(Dodge et al., 2010)
<i>Combinations of GDNF, VEGF, IGF-1 and/or BDNF</i>								
t.p.: GM	GM	<i>Ex vivo</i> MPC (2.5x10 ⁵ /each NTF)	CMV	B6.SOD1 ^{G93A} mouse	P90	9.3%	Combined MPC preserved NMJ (88%), rescued MNs axons (6-fold) and delayed onset (52%). Reduced benefits from single MPC-NTF.	(Dadon-Nachum et al., 2014)
t.p.: TA + TB + LT	Skeletal muscles	<i>Ex vivo</i> LV MPC; 3 times (1.5x10 ⁵ /site.)	PGK1	SD SOD1 ^{G93} rats	P90	10.4%, 7.5%, 16.2%	GDNF and VEGF prolonged survival (10.4 and 7.5%), and VEGF delayed onset (5.8%). Both slowed BBB scores, preserved NMJ and MNs, to a larger extent when combined. GDNF+VEGF also delayed onset and extended survival (16.2%).	(Krakora et al., 2013)

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
EAAT2, GDH2 and NRF2								
i.c.m. + i.m.: GM	Upper and lower MNs	LV (5x10 ⁸ vp)	CMV	B6.SOD1 ^{G93A} mouse	P65	19-22d	Only the combination improved NS, weight loss, hindlimb reflex, motor function and delayed onset. Increased in 2-fold MN numbers.	(Benkler et al., 2016)
OTHERS								
NRF2, PRDX3								
i.m.: FCM + TG + InC + DiA + HL	Skeletal muscles	AAV6 (1.2x10 ¹¹ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P30	0%	Neither showed differences in NS, disease onset, rotarod performance, survival or MN death.	(Nanou et al., 2013)
IL-10								
i.s.: lumbar	Spinal cord	AAV1 (N/A)	N/A	B6SJL.SOD1 ^{G93A} mouse	P0	15%	No changes in body weight or in GFAP ⁺ or Iba1 ⁺ cells, but the upregulation of a subset of innate immunity genes was attenuated.	(Ayers et al., 2015)
DOK7								
i.v.: tail	NMJ	AAV9 (1.2x10 ¹² vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P90	7.5%	Enhanced MuSK activation and clustered AChR in NMJ. Protected from nerve and muscle atrophy, but not from MNs loss.	(Miyoshi et al., 2017)
MCP1								
IM: TA + GM + GLU + TB	Skeletal muscles	scAAV9 (2.18x10 ¹¹ vg/site)	CMV	C57BL/6SOD1 ^{G93A} ; 129SvSOD1 ^{G93A} mice	P42; P56	N/A	Promoted axonal regeneration and prevented inflammation and MN loss. Disease progression and onset were ameliorated.	(Trolese et al., 2022)
Caveolin-1								
s.p.: lumbar	MNs	scAAV9 (2x10 ¹¹ vg)	SYN	C57BL/6.SOD1 ^{G93A} mouse	P56	10%	Delayed disease onset, greater running-wheel performance, preserved MNs and NMJ integrity.	(S. Wang et al., 2022)
s.p.: cervical	MNs	scAAV9 (4x10 ¹¹ vg)	SYN	SD SOD1 ^{G93A} rats	2 mo	N/A	Preserved forelimb grip strength and MEPS in the brachial and gastrocnemius muscles.	-

Table 12. Gene silencing and editing strategies tested in ALS models

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
shRNA / siRNA								
SOD1								
i.m.: HL + FCM + TG + DiA + InC	Skeletal muscles	RGP-LV (total of 120 μ l, 0.7-1x10 ⁹ TU/ml)	H1	B6SJL.SOD1 ^{G93A} mouse	P7	77%	Strong protection of MNs and improved motor performance, resulting in a delay in the onset by more than 100%.	(Ralph et al., 2005)
i.s.: bilateral	Lumbar spinal cord	LV (90 ng p24 antigen/ site)	H1	B6SJL.SOD1 ^{G93A} mouse	P40	0%	Retards onset and progression rate of the disease. Preserved MNs and GM CMAP amplitude.	(Raoul et al., 2005)
i.t. pump	CNS	Modified siRNA	-	B6SJL.SOD1 ^{G93A} mouse	P85	6%	Widespread distribution siRNA, knocked down mSOD1 expression and slowed the disease progression.	(Wang et al., 2008)
i.c.: 16 sites motor cortex	UMNs	AAV9 (N/A)	H1	SD SOD1 ^{G93A} rat	P70	12%	Significant delay of disease onset and enhanced survival of LMNs and maintenance of NMJs.	(Thomsen et al., 2014)

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
s.p.: lumbar + cervical	CNS	scAAV9 (5.6x10 ¹⁰ vg)	H1	B6SJL.SOD1 ^{G37R} mouse	P120	Corrected	Treatment before onset prevents MND. Preservation of MEP, α-MNs and interneurons. Suppression of peripheral denervation. No spinal atrophy and misfolded SOD1, and no changes in inflammation or gene expression.	(Bravo-Hernandez et al., 2020)
s.p.: lumbar + cervical	CNS	scAAV9 (5.6x10 ¹⁰ vg)	H1	B6SJL.SOD1 ^{G37R} mouse	P348	Corrected	Treatment after onset stops progression, partly preserving strength, escaping paralytic state and preserving MNs.	-
i.c.m	Spinal cord	scAAV9 (8x10 ⁹ vg/g)	H1	B6SJL.SOD1 ^{G93A} mouse	P1	42%	First report to show no significant off-targets. 88% decrease in MNs death. Improved motor and neurological deficits. Decreased gliosis and CSF hSOD1 levels.	(Iannitti et al., 2018)
i.c.m	Spinal cord	scAAV9 (5x10 ¹⁰ vg/g)	H1	B6SJL.SOD1 ^{G93A} mouse	P40	13.8%	Longer latency to fall from rotarod. Decreased body weight loss and enhanced neuroscoring.	-
i.v.: temporal vein	MNs	AAV9 (3.6 × 10 ¹⁴ vg/kg)	H1	B6SJL.SOD1 ^{G93A} mouse	P1	39%	Treatment at all ages improved motor function and retained muscle mass. Only P1 delayed disease onset (38%), due to more efficient MNs SOD1 silencing.	(Foust et al., 2013)
i.v.: tail vein	MNs	AAV9 (1.7x10 ¹⁴ vg/kg)	H1	B6SJL.SOD1 ^{G93A} mouse	P21	29.5%	P21 injection slowed disease progression compared to P1, which had less transduced non-neuronal cells.	-
i.v.: tail vein	MNs	AAV9 (1.6x10 ¹⁴ vg/kg)	H1	B6SJL.SOD1 ^{G93A} mouse	P85	22.7%	Treatment improved motor function and retained muscle mass.	-
i.v.: tail vein	MNs	AAV9 (1.1x10 ¹⁴ vg/kg)	H1	B6SJL.SOD1 ^{G37R} mouse	P215	22%	Treatment after onset slowed disease progression, preserving weight and force.	-
i.v.: tail vein	Broad	AAV6 (2x10 ¹¹ vg)	H1	B6SJL.SOD1 ^{G93A} mouse	P42	0%	No benefit was conferred.	(Towne et al., 2008)
i.m.: HL + FL + InC + FCM + TC + TG	MNs	AAV6 (2x10 ⁷ TU/site, TC: 8x10 ⁷ TU)	H1	B6SJL.SOD1 ^{G93A} mouse	P1-5,	0%	Protected transduced MNs and myofiber from atrophy, decreased NMJ denervation. No changes in glial reactivity. Delayed loss of TS CMAP and increased weight, but swimming latency was unaltered.	(Towne et al., 2011)
i.n.: SCN	Spinal MNs	rAd, AAV2 (1.25x10 ⁹ vp)	U6	B6SJL.SOD1 ^{G93A} mouse	P94	7.5%	rAd increased survival and attenuated weight loss, but not AAV2, which targeted fewer MNs.	(Wu et al., 2009)
FUS								
i.c.: right MC	Cortical MNs	AAV9 (1x10 ¹¹ vg)	H1	NHP	57, 73 mo.	N/A	~80% KO across cell types. MC Iba1+ and GFAP+ signal was higher in shFUS-treated cortex than in control-injected left cortex.	(Endo et al., 2018)
miRNA								
SOD1								
i.l. + i.p.	Respiratory MN	AAVrh10 (1x10 ¹¹ vg)	H1	B6SJL.SOD1 ^{G93A} mouse	P60	36%	Preserved diaphragm NMJ and axon number in phrenic and hypoglossal nerves, but not MNs. Enhanced breathing but did not ameliorate lung phenotype.	(Keeler et al., 2020)
i.t.: lumbar infusion	MNs	AAVrh10 (3.5x10 ¹³ vg)	CBA, H1, U6	NHP	N/A	N/A	In MNs silencing correlated with promoter strength (CBA<H1-U6), greater in lumbar regions. Preexisting NAb did not affect distribution or silencing. No off-targets and NAb responses.	(Borel et al., 2018)
i.t. lumbar 2 speeds	Spinal cord	AAVrh10 (2.4x10 ¹⁰ vg; 8 min vs 30s)	CBA	B6SJL.SOD1 ^{G93A} mouse	P30	8.8%; 5.3%	Slow and fast injections tended to delay onset (11.7 vs 8.1%) and extend survival, better for slow injections, which displayed longer footprint and slowed weight loss.	(Li et al., 2017)

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
i.c.v.: lateral ventricles	CNS	AAV9 (5x10 ¹⁰ vg/ventricle)	CBA	B6SJL.SOD1 ^{G93A} mouse	P0-1	50%	Reduction of mSOD1 mRNA in UMNs and LMNs. Preserved numbers of LMNs, diameter of axons, and attenuated inflammation. Delayed hindlimb paralysis.	(Stoica et al., 2016)
i.v.: tail; i.t.: lumbar (NHP)	Spinal cord	AAVrh10 (IV:2x10 ¹¹ vg; IT:6x10 ¹² vg/kg)	U6, CB	B6SJL.SOD1 ^{G93A} mouse; NHP	P50-P68; <4 yrs.	20%	In mice, treatment delayed disease onset and extended survival by ~20% for both promoters. It preserved limb strength, motor skills and inspiratory muscle strength. In NHP, 21% (CB) or 93% (U6) silencing were achieved in lumbar MNs.	(Borel et al., 2016)
i.c.v.: lateral left ventricle	MNs	AAV6 (1.6x10 ¹¹ vg);	CMV	(SOD1 ^{G93G}) ^{dl} mouse	P2	26%	AAV6-CMV corrected TS CMAP and swimming performance. Prolonged disease onset (25%). Spinal MNs, muscle integrity and NMJ were preserved.	(Dirren et al., 2015)
i.c.v.: lateral left ventricle	Astrocytes	AAV9 (6.8x10 ¹¹ vg);	gfaABC ₁ D	(SOD1 ^{G93G}) ^{dl} mouse	P2	14%	AAV9-gfaABC ₁ D corrected TS CMAP and swimming performance. Spinal MNs, muscle integrity and NMJ were preserved.	-
i.c.v.: left lateral	MNs and astrocytes	AAV6 (8x10 ¹¹ vg) + AAV9 (3.41x10 ¹¹ vg);	CMV; gfaABC ₁ D	(SOD1 ^{G93G}) ^{dl} mouse	P2	10%	The combination corrected TS CMAP, swimming performance and median survival. Prolonged disease onset (12.5%). Spinal MNs, muscle integrity and NMJ were preserved.	-
i.t.: lumbar	MNs	AAV9 (2.4x10 ¹² vg),	CMV	(SOD1 ^{G93G}) ^{dl} mouse	P35	0%	Preserved MNs and NMJ improved TS CMAP and motor function. Disease onset, progression and survival were not affected.	-
i.t.: lumbar	Astrocytes	AAV9 (2.4x10 ¹² vg),	gfaABC ₁ D	(SOD1 ^{G93G}) ^{dl} mouse	P35	0%	Improved TS CMAP and motor function, preserved lumbar MNs and NMJ. Disease onset, progression and survival were not affected. Targeting MNs showed better outcomes.	-
i.t.: lumbar	MNs	AAVrh10 (2.4x10 ¹⁰ vg)	CBA	B6SJL.SOD1 ^{G93A} mouse	P65	13.9%	Did not alter disease onset but slowed progression. Extended survival, correlating with transduction.	(Wang et al., 2014)
i.c.v.: left lateral	Astrocytes	AAV9 (4.2x10 ¹¹ vg)	gfaABC ₁ D	B6SJL.SOD1 ^{G93A} mouse	P2	N/A	Rescues NMJ by collateral sprouting, preserves fast-fatigable MU, GM CMAP amplitude and motor function.	(Rochat et al., 2022)
C9ORF72								
i.s.t.	Brain	AAV5 (5x10 ¹⁰ or 1x10 ¹⁰ vg)	CAG	Tg(C9orf72_3) line 112	P90	N/A	20%–40% lowering of C9orf72 mRNA and 20% less cells containing RNA foci.	(Martier et al., 2019)
miR-17~92								
i.t.: lumbar	Spinal MNs	scAAV9 (1x10 ¹¹ vg)	CMV	B6SJL.SOD1 ^{G93A} mouse	P60	14%	miR-17~92/PTEN axis is related to MN degeneration and innervation, being dysregulated in SOD1 ^{G93A} mice. Overexpression slightly ameliorated GM CMAP.	(Tung et al., 2019)
ASO								
SOD1								
i.c.v. pump	CNS	ASO (100 µg/d)	-	SD SOD1 ^{G93A} rat	P65-75	8	Reduced SOD1 protein and mRNA in brain and spinal cord. Significantly slowed disease progression.	(Smith, 2006)
i.t. pump	CNS	ASO (0.15 mg, 0.5 mg, 1.5 mg, 3 mg /12h)	-	Human	Sym p.	0	First-in-human clinical phase I trial. Safe, no dose-limiting toxicities. Re-dosing was well tolerated. Dose-dependent CSF ASO concentrations.	(Miller et al., 2013) ClinicalTrials.gov: NCT01041222
i.t. pump	CNS	ASO (20-100 mg/ over 12w)	-	Human	Sym p.	0	Phase I/II clinical trial. Dose-dependent CSF SOD1 reduction, 33% in the highest group. Decreased rate of decline in ALSFRS-R.	(Miller et al., 2020) ClinicalTrials.gov: NCT02623699

Delivery	Target	Vector (dose)	Promoter	Model	Age	Survival	Main findings	Reference
i.c.v.	CNS	ENA-ASO (200 mg)		hTDP-43 mice	P42	N/A	Suppresses the formation of cytosolic TDP-43 inclusions, normalizes weight, anxiety-like behavior, and locomotion but not the strength.	(Takeuchi et al., 2023)
CRISPR								
Cas9-SOD1								
i.v.: facial vein	Broad	AAV9-2YF (4 × 10 ¹¹ vg)	CMV U6-sgRNA	B6SJL.SOD1 ^{G93A} mouse	P0- P1	25%	Reduced mSOD1 protein by >2.5-fold in the spinal cord. ~50% more MNs. Improved motor function, reduced muscle atrophy and delayed onset (37%)	(Gaj et al., 2017)
i.c.v.: lateral ventricle	CNS	AAV9 (1.2x10 ¹¹ vg)	CMV U6-sgRNA	B6SJL.SOD1 ^{G93A} mouse	P0	22%	Delayed onset and prolonged survival, but disease duration was shortened. Hindlimb stretch and stride length were improved. Decreased Iba1 ⁺ cells and hSOD1, increased spinal MNs.	(Duan et al., 2020)
i.c.v.: lateral ventricles	CNS	AAV-PHP.B (2x10 ¹¹ vg)	U6-sgRNA	B6SJL.H11 ^{Cas9} SOD1 ^{G93A} mouse	P0	66 and 85%	Prevents muscle atrophy and NMJ and MN loss. Prevents motor function decline.	(Chen et al., 2023)
i.t.	CNS	AAV-PHP.eB (1x10 ¹² vg)	U6-sgRNA	B6SJL.H11 ^{Cas9} SOD1 ^{G93A} mouse	P35	≥ 200%	Fully preserves neuromuscular function. Prevented body weight loss and TA CMAP decline. ~80% SOD1 reduction in the spinal cord and cortex.	-
i.v.	Broad	AAV-PHP.eB (1x10 ¹² vg)	U6-sgRNA	B6SJL.H11 ^{Cas9} SOD1 ^{G93A} mouse	P35	≥ 200%	Same favorable outcomes as i.t. administration. Animals had to be euthanized at P340.	-
RfxCas13d-SOD1								
i.t.: lumbar	CNS	AAV9 (2x10 ¹¹ vg)	CAG- U6-sgRNA	B6SJL.SOD1 ^{G93A} mouse	P60- 65	65%	Strong hSOD1 depletion. Improves rotarod performance, disease duration and body weight loss	(Powell et al., 2022)
CBE-hSOD1								
i.t.: lumbar	Spinal cord	Dual AAV9 (8x10 ¹⁰ vg)	CAG	B6SJL.SOD1 ^{G93A} mouse	P56- 60	11%	Disease onset not delayed. Improved rotarod and grip strength. Less SOD1 inclusions and astrogliosis. Rescue of MNs and denervation of NMJ.	(Lim et al., 2020)

AAV: adeno-associated virus, AMPA: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid, BBB score: Basso-Beattie-Bresnahan locomotor rating score, Cb: combined, CB: cytomegalovirus enhancer/ β -actin promoter, CBA: chicken beta-actin, CBE: cytosine base editor, CMV: cytomegalovirus promoter, CT-1: cardiotrophin 1, DAO: D-amino acid oxidase, DCN: deep cerebellar nuclei, FMN: facial motor nucleus, DIA: diaphragm LT: longissimus thoracis, FC: facial muscles, FL: forelimb, G-CSF: granulocyte-colony stimulating factor, GDNF: glial cell-derived neurotrophic factor, GLT1: glial glutamate transporter 1, GLU: gluteus maximus, GluA2: subunit of the AMPA receptor, GM: gastrocnemius, HGF: hepatic growth factor, HGF: hepatocyte growth factor, HL: hindlimb, i.c.: intracerebral, ICap: internal capsule, i.c.b.: intracerebellar, i.c.m.: intracisterna magna, i.c.v.: intracerebroventricular, IGF-1: insulin-like growth factor, i.l.: intralingual, InC: intercostal muscles, i.p.: intrapleural, i.s.: intraspinal, i.s.t.: intrastratral, LV: lentivirus, MC: motor cortex, MIF: macrophage migration inhibitory factor, MMP: measurement of mitochondrial function, MPC: muscle progenitor cells, MSC: mesenchymal stem cells, MuSK: muscle-specific receptor tyrosine kinase, NAb: neutralizing antibodies, NPC: neural progenitor cells, NRG1-I: neuregulin type I, NRG1-III: neuregulin 1 type III, NS: neurological score, NT-3: neurotrophin-3, p-NF: phospho-neurofilament, PGK1: phosphoglycerate kinase -1, PhMNs: phrenic motoneurons, pmn: progressive motor neuronopathy, PSM: paraspinal muscles, QD: quadriceps, RSV LTR: Rous sarcoma virus long terminal repeat promoter, SD: Sprague Dawley rats, SFP: spontaneous fibrillation potentials, SYN: synapsin I, SYT13: synaptotagmin 13, TA: tibialis anterior, TB: triceps brachii muscles, TC: thoracic muscles, TG: tongue, t.p.: transplant, TS: triceps surae muscle, TU: transducing units, CTNF: ciliary neurotrophic factor.

PATENT PUBLICATION

Treatment of neuromuscular diseases via gene therapy that
expresses Klotho protein

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2023/198828 A1

(43) International Publication Date
19 October 2023 (19.10.2023)

(51) International Patent Classification:

A61K 38/47 (2006.01) A61P 25/28 (2006.01)
A61K 48/00 (2006.01) C12N 9/24 (2006.01)
A61P 21/00 (2006.01) C12N 15/861 (2006.01)

(21) International Application Number:

PCT/EP2023/059677

(22) International Filing Date:

13 April 2023 (13.04.2023)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/330,684 13 April 2022 (13.04.2022) US

(71) Applicants: **UNIVERSITAT AUTÒNOMA DE BARCELONA** [ES/ES]; Edifici A. Rectorat, Campus universitari s/n, 08193 BELLATERRA (ES). **CONSORCIO CENTRO DE INVESTIGACIÓN BIOMÉDICA EN RED** [ES/ES]; Av. Monforte de Lemos 3-5. Pabellón 11, 28029 MADRID (ES). **INSTITUCIÓ CATALANA DE RECERCA I ESTUDIS AVANÇATS** [ES/ES]; Pg. Lluís Companys, 23, 08010 BARCELONA (ES). **FUNDACIÓ HOSPITAL UNIVERSITARI VALL D'HEBRON - INSTITUT DE RECERCA** [ES/ES]; Passeig Vall d'Hebron, 119-129, 08035 BARCELONA (ES).

(72) Inventors: **BOSCH MERINO, Assumpció**; C. Martí L'Humà 7, 08195 SANT CUGAT DEL VALLÈS (ES). **VERDÉS FRANQUESA, Sergi**; C. Tapióles 11, 1er pis, 25210 GUISSONA (ES). **HERRANDO GRABULOSA, Mireia**; C. Galejadors, 7, 08540 CENTELLES (ES). **NAVARRO ACEBES, Xavier**; Universitat Autònoma de Barcelona, Dept. Biologia Cel·lular, Fisiologia i Immunologia, 01893 BARCELONA (ES). **CHILLON RODRIGUEZ, Miguel**; C. Martí L'Humà 7, 08195 SANT CUGAT DEL VALLÈS (ES).

(74) Agent: **ZBM PATENTS - ZEA, BARLOCCI & MARKVARDSEN**; Rambla de Catalunya, 123, 08008 Barcelona (ES).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY,

MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

Published:

— with international search report (Art. 21(3))
— with sequence listing part of description (Rule 5.2(a))

WO 2023/198828 A1

(54) Title: TREATMENT OF NEUROMUSCULAR DISEASES VIA GENE THERAPY THAT EXPRESSES KLOTHO PROTEIN

(57) Abstract: The present disclosure provides gene constructs containing a nucleic acid sequences encoding a mammalian s-KL, operatively linked to a muscle cell-specific promoter, for use in the treatment of motor impairment that may manifest, for example, in a neuromuscular disorder or disease, utilizing viral and non-viral vectors with muscle cell and motor neuron tropisms containing gene constructs containing a nucleic acid sequence encoding a mammalian s-KL, operatively linked to a promoter such as a muscle cell-specific promoter, is delivered in pharmaceutical compositions containing the expression vector, isolated cells containing the expression vector, and methods of treating motor impairment and motor neuron diseases.